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Action of Clofazimine on *Mycobacterium avium* and *Mycobacterium intracellulare*

by

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ABSTRACT

Clofazimine is a member of the phenazine pigment family which has been successfully used in chemotherapy against a variety of mycobacteria including *M. avium*. The presence of clofazimine in growth medium resulted in higher carotenoid pigmentation in *M. avium* cells. Carotenoid pigments have been shown to quench superoxide radicals supporting the hypothesis that pigmentation possibly protected cells against superoxide. Clofazimine caused the generation of superoxide radicals in *M. intracellulare* strain LR163 represented by cyanide-resistant oxygen consumption. The amount of oxygen consumed was dependant upon the clofazimine concentration. This supports the hypothesis that clofazimine is antibiotic via its ability to generate toxic oxygen metabolites. Higher catalase activity was found in extracts of cells grown in the presence of a low concentration of clofazimine. At a higher concentration, the amount of catalase and superoxide dismutase activities were lower than the basal level. This finding did not agree with the hypothesis. At this point the reason for the drop in the activities (i.e. lower than basal level) is not known. Clofazimine was mildly synergistic with rifampicin. This result supports hypothesis that the defense mechanism of *M. intracellulare* to clofazimine was enzymatic. Clofazimine-resistant derivatives of *M. intracellulare* strain LR163 have been isolated. Their characterization will provide a direct approach towards determining the mode of action of clofazimine in cells.

Abstract

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INTRODUCTION AND REVIEW OF LITERATURE

THE MYCOBACTERIA

The genus *Mycobacterium* is a heterogenous group of slow and fast growing, pathogenic and non-pathogenic bacteria, all of which can be characterized by their acid-fast staining reaction. In 1882, Koch discovered the best known species of this genus, *Mycobacterium tuberculosis*, the causative agent of tuberculosis. With the decline in the incidence of tuberculosis in the early 1950's, the importance of other pathogenic mycobacteria (called non-tuberculous) was recognized.

Representatives of *Mycobacterium avium, M. intracellulare and M. scrofulaceum* (called the MAIS group) have been isolated from water (Falkinham et al., 1980), aerosol (Wendt et al., 1980) and soil samples (Brooks et al., 1984). Both the frequency of MAIS recovery (Falkinham et al., 1980) or MAIS numbers (Brooks et al., 1984) are higher in southeastern than northeastern United States waters. That distribution coincides with the higher frequency in immune responses to MAIS antigens (Edwards et al., 1969; Edwards, 1970). MAIS organisms are slow growing, opportunistic pathogens and *M. avium* and *M. intracellulare* (called the *M. avium* complex or MAC) in particular, cause pulmonary infections (mycobacteriosis) in humans (Wolinsky, 1979). In the absence of evidence of person-to-person transfer, the source of human MAIS infection was postulated to be environmental (Wolinsky, 1979). This is supported by the fact that MAIS isolates from patients with mycobacteriosis and those from natural aerosols share common characteristics (Meissner and Falkinham, 1986; Fry et al., 1986).

Recently it has been shown that up to 25% of patients with either acquired immunodeficiency syndrome (AIDS; Zakowski, et al., 1982; Winter, et al., 1985; Wong, et al., 1985) or Kaposi's sarcoma (Fainstein, et al., 1982) or under immunosuppressive drug treatment (Simpson, et al., 1982) have disseminated infections with *M. avium* complex. Because patients with AIDS frequently have disseminated *M. avium* complex infections (Blaser and Cohn, 1986) and these infections can be life-threatening (Greene, et al., 1982; Young, 1988), much attention has been placed upon the treatment of these infections (Young, 1988). A major barrier to effective chemotherapeutic treatment of these infections has been the characteristic antibiotic resistance of these organisms (Wolinsky, 1979; Greene, et al., 1982; Wong, et al., 1985; Iseman, et al., 1985; Young, et al., 1986; Young, 1988). To date, other than the suggestion that mycobacterial antibiotic resistance may be due to antibiotic permeability barriers (Rastogi, et al., 1981; Mizuguchi, et al., 1983), the mechanism(s) of resistance are unknown.

ANTIBIOTICS

Clofazimine (i.e. B663, Lamprene) is a member of the phenazine pigment family (Flanzblau and O'Sullivan, 1988). It has been successfully used in chemotherapy against a variety of mycobacteria including *M. avium* (Lindholm-Levy and Heifets, 1988; Young, 1988). Pyocyanine, another member of the phenazine pigment family, is an antibiotic pigment produced by *Psuedomonas aeruginosa* (Hassan and Fridovich, 1980). Phenazines such as pyocyanine have been shown to be more effective under aerobic conditions to produce the superoxide radical O_2^- (and consequently H_2O_2 spontaneously) by the non-enzymatic oxidation of NADH (Hassan & Fridovich, 1980). Paraquat (a phenazine pigment) was shown to induce cyanide-resistant oxygen consumption and induction of the manganese-containing superoxide dismutase in *Escherichia coli* (Hassan and Fridovich, 1979). The activity of clofazimine and other phenazine pigments against *M. tuberculosis* was diminished at low oxygen tensions (Winder, 1982). Catalase-negative strains of *M. tuberculosis* were more susceptible to clofazimine than their catalase-positive parents (Winder, 1982). Pyocyanine-induced superoxide dismutase and catalase in *Escherichia coli* (Hassan et al, 1980), suggesting that either one or both of these enzymes provides protection against the inhibitory action of the phenazines. In addition to the production of catalase and superoxide dismutase by *M. avium*, the yellow carotenoid pigments of *M. avium* strains isolated from patients with AIDS (Kiehn, et al., 1985), might protect strains by their ability to quench O_2^- (Krinsky, 1979; Burton and Ingold, 1984).

Although clofazimine is used in the treatment of MAC infections, its mode of action remains unknown. Clofazimine-resistant mutants of *M. tuberculosis* and *M. smegmatis* have been isolated, though not characterized (David, et al., 1987). However clofazimine-resistant mutants could not be isolated from *M. aurum* (David, et al., 1987). In that organism, colonies recovered from medium containing clofazimine lost their clofazimine-resistance when grown in the absence of the antibiotic (David, et al., 1987). This suggests that they had undegone some adaptation to clofazimine tolerance. That adaptation could have been due to induction of catalase, superoxide dismutase, or pigment. The observation that catalase activity in *M. avium* is induced by aeration and that higher catalase activity results in higher H_2O_2 resistance (Pethel and Falkinham, 1989) supports that possibility.

Thus, there is the suggestion that clofazimine is antibiotic by its ability to generate superoxide (O_2^-) and possibly hydrogen peroxide (H_2O_2). Resistance to clofazimine could therefore occur by induction of catalase, superoxide dismutase, or pigmentation. This could be confirmed by exposing *M. avium* complex cells to clofazimine and measuring (1) pigmentation and (2) superoxide dismutase and (3) catalase activities and (4) the production of superoxide by cells (detected as cyanide-resistant oxygen consumption).

If clofazimine increased pigment and (or) catalase and (or) superoxide dismutase activities as a defense mechanism, addition of any antibiotic which prevents protein synthesis (e.g. streptomycin or rifampicin) would make the cells more susceptible to clofazimine by preventing the induction of pigment, superoxide dismutase or catalase activities, thereby decreasing the minimum inhibitory concentration (MIC) value. The phenomenon of synergism was thus studied by adding the antibiotics rifampicin or streptomycin to cells growing in the presence of clofazimine. A drug combination was considered synergistic if there was no growth of cells in the tube containing the combination but growth in both tubes containing the single antibiotic at one-fourth the MIC (Yajko, et al., 1988).

OBJECTIVES

(1) Develop a method for the measurement of clofazimine in culture medium.

(2) Determine whether clofazimine induces pigmentation.

(3) Determine whether clofazimine induces cyanide-resistant oxygen consumption.

(4) Determine whether clofazimine induces catalase.

(5) Determine whether clofazimine induces superoxide dismutase.

(6) Determine whether clofazimine is synergistic with rifampicin or streptomycin.

(7) Isolate clofazimine-resistant MAIS mutants.

MATERIALS AND METHODS

BACTERIAL STRAINS

Mycobacterium avium strain 2812(P) (Stormer and Falkinham, 1989) and *Mycobacterium intracellulare* strain LR163 (Crawford, et al., 1985) were used in this study.

MEDIA

The mycobacterial cultures were grown in Middlebrook 7H9 broth medium (BBL Microbiology Systems, Cockeysville, MD) containing 0.5% (vol/vol) glycerol and 10% (vol/vol) oleic acid-albumin enrichment (OA) (MGE broth). OA was prepared as described by Pethel and Falkinham, (1989). Stock cultures were grown in 2 ml MGE broth to mid-log phase (10 days) at 37°C in screw-cap tubes (18x150 mm), vortexed daily for aeration and then stored at 4°C. Inocula for larger cultures was 10% of the final culture volume.

The agar medium used was Middlebrook 7H10 (BBL, Microbiology Systems, Cockeysville, MD) containing 0.5% (vol/vol) glycerol and 10% (vol/vol) OA (MGE agar). Plates were sealed with parafilm and incubated at 37°C for three to four weeks. All water used in the preparation of media was glass-distilled.

Plate count agar (PCA, Difco Laboratories, Detroit, MI) was used to detect contaminants. The medium was streaked with a 0.001 ml loop containing culture and incubated at 37°C for 24 hours.

ANTIBIOTICS

Clofazimine (Ciba-Geigy, Suffern, NY) stock solutions were prepared by dissolving clofazimine powder in 100% DMSO in sterile glassware. This solution was sterile (i.e. no colonies on PCA), though not sterilized. The streptomycin stock solution was prepared by dissolving streptomycin sulfate (Sigma Chemical Co., St. Louis, MO) powder in water. This solution was sterilized by autoclaving before use. The rifampicin (Sigma Chemical Co., St. Louis, MO) stock solution was prepared by dissolving rifampicin powder in 100% DMSO in sterile glassware. This solution was sterile (i.e. no colonies on PCA), though not sterilized.

MEASUREMENT OF CLOFAZIMINE IN MEDIUM

The amount of clofazimine in medium was determined by a modification of the method used by Banerjee, et al. (1974). Clofazimine was dissolved at a final concentration of 5 μg clofazimine/ml in 5 ml of M7H9 medium containing 0.5% (v/v) glycerol (MG medium) in screw-cap tubes (18x150 mm). That solution was diluted sequentially by half to give a concentration range from 5.00 to 0.04 μg clofazimine/ml. The control medium lacked clofazimine. Clofazimine was extracted from 3.0 ml medium by adddition of 1.0 ml glacial acetic acid (Sigma Chemical Co., St. Louis, MO) to a final concentration of 25% (v/v) and vortexed (5 sec). Next, toluene (Sigma Chemical Co., St. Louis, MO) was added to a final concentration of 50% (v/v) and the mixture vortexed for 10 sec. The suspension was centrifuged at 4,470 x g for 10 min at 25°C. The upper, 3.0 ml aqueous layer was transferred to a tube containing 2.0 ml of 6N HCl (Sigma Chemical Co., St. Louis, MO). This suspension was vortexed for 10 sec and incubated for 5 min at 25°C. The upper toluene layer was removed and the absorbance (535 nm) of the HCl-extract read. This experiment was performed three times and the data used to construct a standard curve.

EXTRACTION AND MEASUREMENT OF PIGMENT

Cultures of log-phase cells were grown in 100 mls MGE broth containing 0.000, 0.015, 0.030, 0.060 or 0.120 μg clofazimine/ml in 500 ml screw-cap flasks with continuous shaking at 100 rpm (Orbital Shaker Model 3590, Lab Line Instruments, Inc. Melrose Park, IL) at 37°C for 7 days.

Cells were harvested by centrifugation at 7,000 x g for 10 min at 4°C. Each cell pellet was suspended in 5 ml of methanol. Cell suspensions were vortexed (60 sec) and the cell clumps dispersed in a 10 ml tissue homogenizer (Arthur H. Thomas Co., Philadelphia, PA). The suspensions were centrifuged at 7,500 x g for 30 min at 4°C and the supernatant solutions recovered and covered with foil to exclude light. The extraction procedure was repeated by resuspending the cell pellets in 2.5 ml of methanol. Suspensions were vortexed (60 sec), and centrifuged at 7,500 x g for 30 min at 4°C. The supernatant solutions were recovered and covered with foil to exclude light. Supernatants from the two extractions were assayed separately for their pigment content. Absorbance of each methanol extract was read in duplicate at 450 nm for pigmentation and at 540 nm for turbidity (Model 102 Spectrophotometer, Hitachi, Japan). Pigmentation, expressed in absorbance units, was the difference in absorbance at 450 nm and 540 nm (i.e. 450nm - 540nm; Stormer and Falkinham, 1989).

The dry cell weight (mg/ml) for each culture was determined by dispensing (in duplicate), 1.0 ml of each culture before harvest, in a dried, preweighed, aluminum pan. The cell suspension was allowed to dry at 100°C in a heating oven for 24 hours and then weighed. The weight of 1.0 ml uninoculated medium was subtracted from the dried cell culture values.

The carotenoid content of the cells was calculated by determining the absorbances due to the pigment (450nm - 540nm) and the dry weight (mg/ml) of each culture. The extinction coefficient of β-carotene at 450nm used was 2,150 absorbance units per mole/litre based on the assumption that all the pigmentation observed in the extracts was due to β-carotene (Stormer and Falkinham, 1989). Most (> 75%) of the pigment from the cells was extracted in the first 5 ml methanol extraction. The absorbance readings of the first 5 ml methanol-extract used for calculations are reported here.

MEASUREMENT OF OXYGEN UPTAKE

GROWTH AND MEASUREMENT OF DRY WEIGHT OF CELLS

Cultures cells for oxygen uptake studies were grown in 100 ml MGE broth with continuous shaking at 100 rpm (Orbital Shaker Model 3590, Lab Line Instruments Inc. Melrose Park, IL) in 1000 mls screw-cap flasks at 37°C for 6 days (log phase).

Cells were harvested by centrifugation at 7,000 x g for 10 min at 4°C. Cells were washed once in 0.05M potassium phosphate buffer, pH 6.0, by vortexing and pelleted by centrifugation at 7,000 x g for 10 min at 4°C. The cell pellet was resuspended in the same buffer. Cell clumps were dispersed using a tissue homogenizer (Arthur H. Thomas Co., Philadelphia, PA) and by vortexing (30 sec). The suspension was stored at 4°C.

The dry cell weight (mg/ml) for each cell suspension was determined by dispensing (in duplicate), 1.0 ml of each culture before harvest, in a dried, preweighed, aluminum pan. The cell suspension was allowed to dry at 100°C in a heating oven for 24 hours and then weighed. The weight of 1.0 ml medium was subtracted from the dried cell suspension values.

CALIBRATION OF THE OXYGEN MONITORING APPARATUS

The consumption of oxygen was measured using a YSI Model 5331 Oxygen Probe and a YSI Model 53 Biological Monitor (Yellow Springs Instrument Co., Inc., Yellow Springs, OH) and a Clark cell (Gilson Medical Electronic). All assays were carried out at 37°C.

The rate of oxygen consumption was measured by an oxygen electrode covered with a Teflon membrane. The membrane was permeable to oxygen and the amount of oxygen at the cathode depended upon the rate of diffusion of oxygen across the membrane. The rate of diffusion was directly proportional to the amount of oxygen present in the cell suspension.

Because cells were suspended in buffer, it was necessary to determine the dissolved oxygen content of the air-saturated buffer. This was done using a chemical reaction which results in the consumption of oxygen. This reaction consisted of NADH (hydrogen donor), phenazine methosulfate (PMS; hydrogen acceptor), and catalase (cleaves hydrogen peroxide). The reaction resulting in the consumption of oxygen was as follows :

$$PMS + NADH + H^+ \rightarrow PMSH_2 + NAD^+$$

$$PMSH_2 + O_2 \rightarrow PMS + H_2O_2$$

$$H_2O_2 \rightarrow \tfrac{1}{2}O_2 + H_2O$$

$$\text{Net Reaction : } NADH + H^+ + \tfrac{1}{2}O_2 \rightarrow NAD^+ + H_2O$$

The following reaction occurs at the cathode -

$$2H_2O + O_2 + 2e^- \rightarrow H_2O_2 + 2OH^-$$

$$H_2O_2 + 2e^- \rightarrow 2OH^-$$

$$\text{Net Reaction : } 2H_2O + O_2 + 4e^- \rightarrow 4OH^-$$

The following reaction occurs at the anode -

$$4Ag + 4Cl^- \rightarrow 4AgCl + 4e^-$$

As seen from the above reactions, the rate of electron flow was directly proportional to the amount of oxygen available to the cathode. This electron flow generated (i.e. current) in the system was measured by the polarograph.

Solutions for the calibration of the apparatus were prepared as follows. Twenty-five mg of NADH was dissolved in 16 ml of buffer. This solution was diluted 1:100 in buffer and its absorbance measured in a 1 cm quartz cuvette at 338 nm. The precise concentration of NADH in the solution was calculated using Beer's Law ($A = \varepsilon bc$), where A is the absorbance of the solution at 338 nm, ε the extinction coefficient of NADH (6,220), b the path length (1 cm) and c the concentration of NADH in moles/litre. The final concentration for PMS (Sigma

Chemical Co., St. Louis, MO) was 2.2μM and for catalase (Sigma Chemical Co., St. Louis, MO) was 0.61 units/ml in buffer. Stocks of both were dissolved in buffer.

Calibration was done by filling the Clark cell chamber with 1.7 ml of buffer solution containing PMS and catalase. Air was bubbled through this solution for 3 min to saturate it with atmospheric oxygen (i.e. 21% O_2) while stirring the solution with the magnetic stir bar. The oxygen monitor and recorder was adjusted to read 100%. This adjustment allowed the calculation of μl of oxygen consumed by one scale division of recorder paper.

Following saturation, the chamber was sealed and once the readings on the oxygen monitor and the recorder pen were stable, a known amount of the NADH solution was added. The decrease in the number of scale divisions on the recorder paper were recorded. Once O_2 consumption ceased (i.e. all the NADH used up), the procedure was repeated six times and an average value calculated.

The concentration of oxygen in solution was calculated by multiplying the number of μmoles of NADH added by 100 and then dividing this product by [(2 x average recorder deflection) x (Volume of cell chamber)]. To determine the μl of oxygen/ml of buffer, the concentration of oxygen in μmoles calculated was multiplied by 22.4 μl (volume occupied by 1μmole of pure oxygen at standard temperature and pressure).

MEASUREMENT OF OXYGEN CONSUMPTION BY CELLS

Oxygen consumption by cells was measured by filling the chamber with the cell suspension (chamber volume = 1.7 mls). Once the meter reading stopped falling, the chamber was sealed and the endogenous oxygen consumption rate measured over a 10 min period. If the oxygen monitor and recorder values were over 100 % (i.e. saturated with O_2), nitrogen gas was bubbled until the values were near 80%.

Effect on oxygen consumption of glucose or glycerol 3% (w/v final concentrations) or OA at a final concentration of 0.06% (v/v) oleic acid and 5% (w/v) bovine serum albumin were determined. Substrates were added to the cell suspension after the endogenous rate was stable (10 min). After a constant new rate was achieved (7 to 8 min), different volumes of 500 mM KCN were added. The minimum final concentration of KCN needed to completely stop oxygen consumption within 3 to 4 min was 3.8mM.

The effect of incubating the cell suspension at 37°C for 26 hours or at 4°C for 4 days (i.e. starvation) upon the endogenous oxygen consumption rate was determined.

The effect of 23.5, 11.8, 5.9, 2.9, and 1.5 μg clofazimine/ml (final concentration) on cellular oxygen consumption was determined by adding different volumes of the stock clofazimine after an endogenous rate was established. Following clofazimine addition, a new constant rate of oxygen uptake was established (7 to 8 min) and the effect of KCN (3.8 mM final concentration) measured. Because clofazimine was dissolved in DMSO, the effect of addition of DMSO equal in volume to the amount of clofazimine solution added was measured.

In each experiment, the oxygen consumption rates due to : (1) endogenous respiration, (2) substrate addition, and (3) KCN addition were measured sequentially. All experiments were done in duplicate. For all oxygen consumption rate calculations, the rate of change between the fourth and sixth minute was used.

MEASUREMENT OF CATALASE ACTIVITY

Cell cultures for the measurement of catalase activity of cells grown in the presence or absence of clofazimine were grown in 250 ml MGE broth containing 0.00, 0.025 or 0.06 μg clofazimine/ml at 37°C for 6 days in 500 ml screw cap flasks.

Cells were harvested by centrifugation at 10,000 x g for 15 min at 4°C. The cells were washed once in 25 ml of 0.05 M sodium phosphate buffer (pH 7.0) and pelleted by centrifugation at 10,000 x g for 15 min at 4°C. The cell pellets were suspended in 5 ml of the same buffer. Cell clumps were dispersed using a tissue homogenizer (Arthur H. Thomas Co., Philadelphia, PA) and by vortexing. Cell suspensions were kept on ice. The cells were broken by two passages through a cold French Pressure cell (Aminco model J4-3398A; American Instrument Co., Silver Spring, MD) at 18,000 to 20,000 lb/in^2 The crude extracts were clarified by centrifugation at 23,000 x g at 4°C for 30 min, and the supernatant solutions were retained for the enzyme assays and stored at 4°C.

Catalase activity of the supernatants was determined by the method of Beers and Sizer (1952). Protein content of each extract was measured using the method of Lowry et al. (1951), using bovine serum albumin, fraction V (Sigma Chemical Co., St. Louis, MO) as a standard. Catalase activity was expressed as units/mg protein. One unit of catalase activity was equal to the decomposition of 1 μmole hydrogen peroxide/minute. The reaction was not linear beyond 1.5 min, therefore the fall in absorbance in the first minute interval was used in the calculations. The change in absorbance due to the cell extract and the stability of the H_2O_2 over this period were measured as controls.

MEASUREMENT OF SUPEROXIDE DISMUTASE ACTIVITY

To determine the activity of superoxide dismutase (SOD) in cells grown in the presence and absence of clofazimine, cell cultures were grown in 250 ml MGE broth containing 0.00 or 0.06 μg clofazimine/ml at 37°C for 6 days in 500 ml screw cap flasks.

Cells were harvested by centrifugation at 10,000 x g for 15 min at 4°C. The cells were washed once in 25 ml of 0.05 M potassium phosphate buffer (pH 7.8) containing 1 mM EDTA and pelleted by centrifugation at 10,000 x g for 15 min at 4°C. The cell pellets were suspended in 5 ml of buffer and clumps dispersed by vortexing (60 sec). Cell suspensions were kept on ice. The cells were broken by two passages through a cold French Pressure cell (Aminco model J4-3398A; American Instrument Co., Silver Spring, MD.) at 18,000 to 20,000 lb/in^2.

The crude extracts were clarified by centrifugation at 23,000 x g at 4°C for 30 min, and the supernatant solutions were retained for the enzyme assays and stored at 4°C.

SOD activity was assayed by measuring the inhibition of the reduction of nitroblue tetrazolium (NBT) (Sigma Chemical Co., St. Louis, MO) in the presence of phenazine methosulfate (PMS) (Sigma Chemical Co., St. Louis, MO) and NADH (Sigma Chemical Co., St. Louis, MO) by measuring the absorbance at 560 nm as described by Fried (1975). Absorbance values were taken over a 5 min interval. The change in absorbance due to the reduction of NBT by PMS in the presence of NADH (i.e. uninhibited reaction), cell extract (i.e. extract stability), and the spontaneous reduction of NBT in the presence of PMS, but absence of NADH (i.e. stability of reagents), were measured as controls. Protein content of each extract was measured using the method of Lowry et al. (1951), using bovine serum albumin, fraction V (Sigma Chemical Co., St. Louis, MO) as a standard.

A standard curve was constructed using bovine erythrocytes SOD (Sigma Chemical Co., St. Louis, MO) in the presence of the above mentioned reagents. The amount of SOD required to inhibit the reduction of nitro-blue tetrazolium by 50% was defined as 1 unit (U) of activity. From the standard curve, the number of SOD units present in each of the extracts was determined. SOD activity was expressed as units/mg protein.

MEASUREMENT OF ANTIBIOTIC SYNERGISM

To study the possibility of synergism between clofazimine and either streptomycin or rifampicin, cells were grown in the presence of various concentrations of clofazimine, streptomycin and rifampicin. The concentration range used was a two-step dilution series for (1) 1.0 to 0.01 μg clofazimine/ml, (2) 1.0 to 0.01 μg streptomycin/ml, and (3) 0.1 to 0.001 μg rifampicin/ml. For combinations of antibiotics tested, the two-step dilution series of each drug pair were respectively diluted down to the lowest concentration of each antibiotic. Screw-cap tubes (18x150 mm) containing 2 ml M7HSF broth (Yajko, et al., 1988) and the respective antibiotics were inoculated with a 0.2 ml of a 7 day log phase culture to a final concentration of 4.0 x 10^6 colony forming units/ml. These cultures were incubated at 37°C for 7 days and vortexed every 48 hours for aeration.

After 7 days of growth in the presence or absence of antibiotics, the minimum inhibitory concentration (MIC) for each antibiotic alone or in combination was determined. The MIC was that concentration at which no turbidity was observed (Yajko, et al., 1988). The minimum bactericidal concentration (MBC) was determined by spreading (in duplicate), 0.01 ml of each culture on MGE agar. Plates were sealed with parafilm and incubated at 37°C for 12 days. MBC was defined as that concentration at which 99% of the initial inoculum was killed (Yajko, et al., 1988).

Synergism was determined by calculating the fractional inhibitory concentration (FIC) and the fractional bactericidal concentration (FBC) values for each of the drug combinations being studied (Yajko et al., 1988) The following formulae were used to determine these values -

$$FIC = MIC\ (\text{Drug in combination}) \,/\, MIC\ (\text{Drug alone})$$

$$FBC = MBC\ (\text{Drug in combination}) \,/\, MBC\ (\text{Drug alone})$$

Drugs were synergistic if the FBC or the FIC values for each drug combination being studied was less than or equal to 0.5.

ISOLATION OF CLOFAZIMINE-RESISTANT MUTANTS

Cultures for the isolation of clofazimine resistant mutants were grown in 100 ml MGE broth with continuous shaking at 100 rpm (Orbital Shaker Model 3590, Lab Line Instruments Inc. Melrose Park, IL) in 1000 mls screw-cap flasks at 37°C for 6 days (log phase).

Cells were harvested by centrifugation at 7,000 x g for 10 min at 4°C, washed once in 25 ml of distilled water by vortexing and pelleted by centrifugation at 7,000 x g for 10 min at 4°C. The cell pellet was resuspended in water to 3.5×10^{10} cololony forming units/ml (CFU/ml) and 0.001 ml of the cell suspension was streaked on MGE agar and PCA to prove viability and the absence of contaminants, respectively. This suspension was stored at -70°C.

Isolation of clofazimine-resistant, spontaneous mutants was attempted by spreading 0.1 ml of the thawed cell suspension on MGE agar containing 1.0 or 10.0 μg clofazimine/ml.

In addition, mutations were induced by adding (10 μl) ethyl methane sulfonate (Sigma Chemical Co., St. Louis, MO) and a few granules of N-methyl, N′-nitro, N-nitrosoguanidine (NTG; Sigma Chemical Co., St. Louis, MO) on MGE agar containing 1.0 or 10.0 μg clofazimine/ml spread with 0.1 ml of the cell suspension. The mutagens were added to the same medium, separated by 5 cm. All the plates were sealed with parafilm and incubated at 37°C for 22 days.

Plates were observed for growth of single colonies and colonial morphology, pigmentation and size noted. Putative mutants were picked and streaked for purity on MGE agar plates containing 1.0 μg clofazimine/ml. Plates were sealed with parafilm and incubated at 37°C for 15 days. Isolated colonies were suspended in 2 ml MGE broth medium and cultures incubated at 37°C for 45 days. Broth cultures were streaked on MGE agar with and without 1μg clofazimine/ml and plates were sealed with parafilm and incubated at 37 °C for 15 days. Broth cultures were stored at 4 °C.

RESULTS

MEASUREMENT OF CLOFAZIMINE IN MEDIUM

An increase in absorbance at 535 nm was observed with an increase in the amount of clofazimine (Table 1). As seen in Figure 1, the increase in absorbance was directly proportional to clofazinine above 0.31 μg clofazimine/ml. There was no significant absorbance of the clofazimine free medium.

PIGMENT PRODUCTION IN CLOFAZIMINE-GROWN CELLS

The amount of pigment extracted from strain 2812P grown in the presence of 0.015, 0.030, and 0.120 μg clofazimine/ml (Table 2) was significantly higher ($0.05 < P < 0.10$) than that extracted from clofazimine-free grown cells. The amount of pigment extracted from cells grown in the presence of 0.06 μg/ml clofazimine was not significantly higher (0.05 < P <

Table 1. Measurement of clofazimine in medium

Clofazimine [a] µg/ml	Absorbance [b] 535 nm
5.00	0.308 ± 0.03
2.50	0.129 ± 0.01
1.25	0.061 ± 0.01
0.63	0.024 ± 0.00
0.31	0.011 ± 0.00
0.16	0.004 ± 0.00
0.08	0.003 ± 0.00
0.04	0.009 ± 0.00
0.00	0.000 ± 0.00

[a] Concentration of clofazimine in medium

[b] Absorbance (535 nm) ± standard deviation

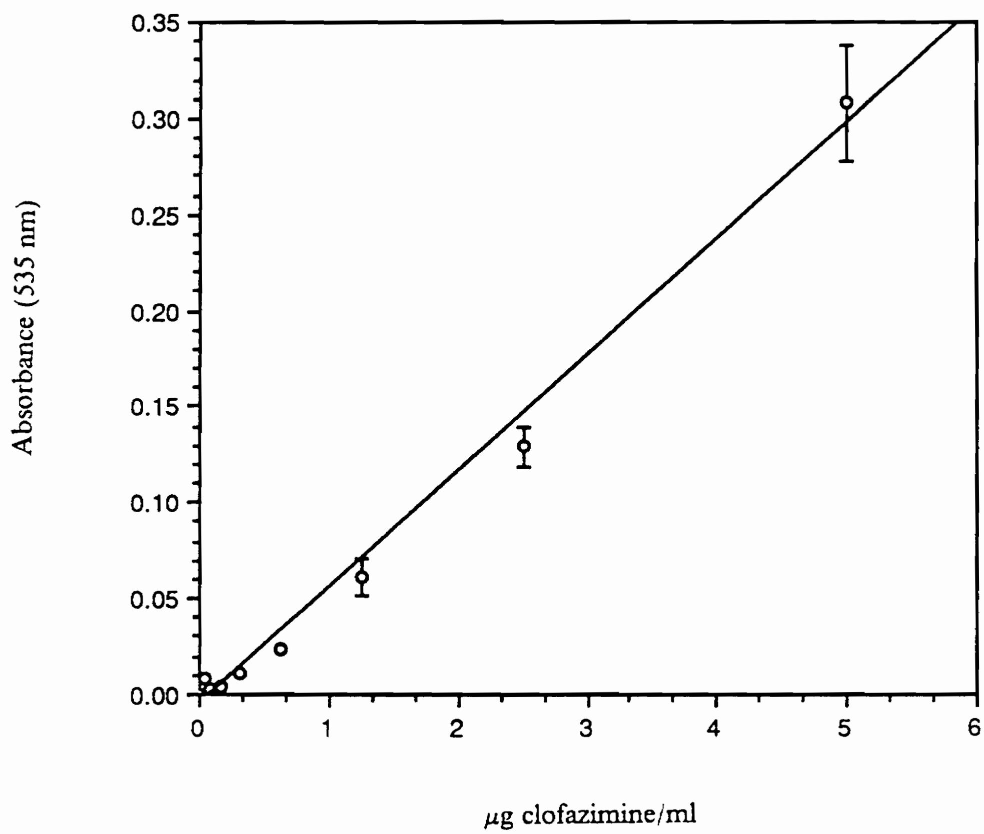

Figure 1. Standard curve for the measurement of clofazimine in medium.

Table 2. Amount of pigment produced by *M. avium* strain 2812P in the presence and absence of clofazimine

Clofazimine [a] μg/ml	Culture Dry weight mg/ml	β-carotene μg/mg D.W.
0.000	110	0.82
0.015	30	3.17
0.030	80	1.35
0.060	110	1.07
0.120	50	2.80

[a] Concentration of clofazimine in MGE medium

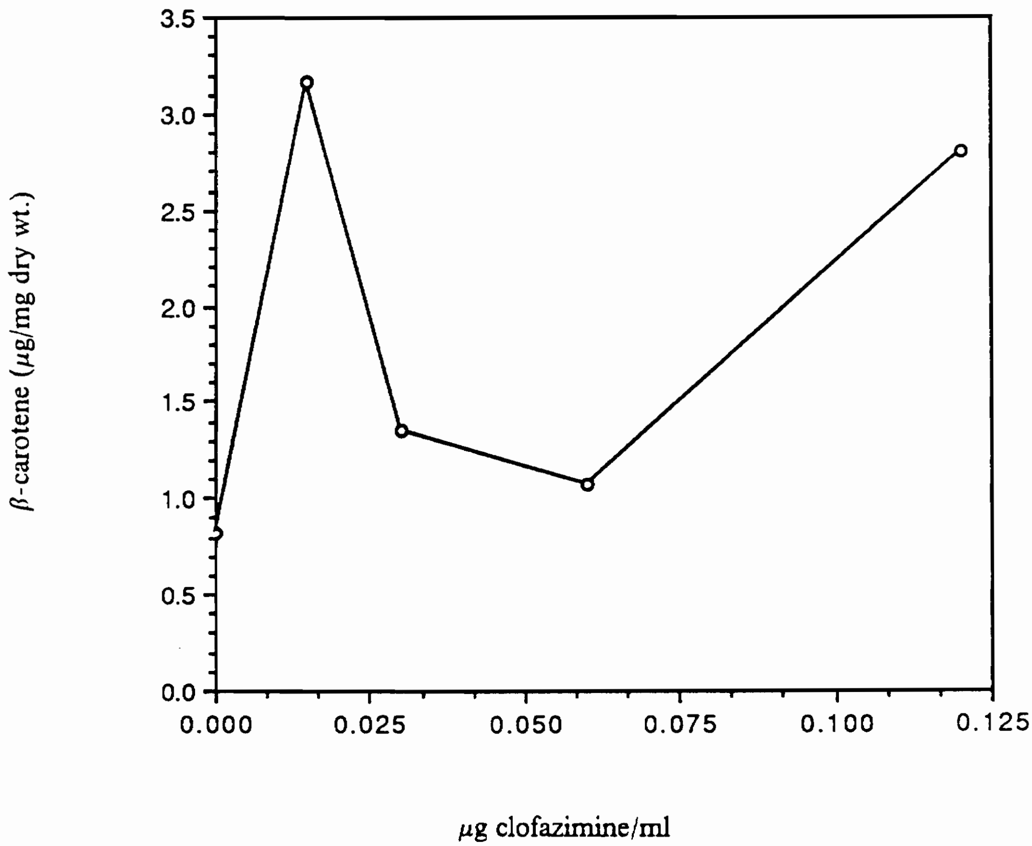

Figure 2. Amount of pigment produced by *M. avium* strain 2812P in the presence of clofazimine.

0.10) (Figure 2). The dry cell weight values showed that there was inhibition of growth in the presence of 0.015, 0.030, and 0.120 μg clofazimine/ml, but none was observed at 0.06 μg clofazimine/ml. The results shown are for a single experiment, but are representative of replicates.

CLOFAZIMINE-INDUCED, CYANIDE-RESISTANT OXYGEN CONSUMPTION

CALIBRATION OF THE OXYGEN MONITORING APPARATUS

For the calibration of the apparatus, NADH (3.1 μmoles/ml) was added to the Clark cell chamber as described in Materials and Methods. The air saturated buffer contained 10 μl oxygen/ml of buffer.

RESPIRATION

Addition of glucose or glycerol (Figure 3) to *M. intracellulare* strain LR163 cell suspension at a final concentration of 0.3% (w/v and v/v repectively) did not stimulate oxygen consumption (Figure 4). OA [final concentration 0.06% (v/v) oleic acid and 5% (w/v) bovine serum albumin] added to the cells led to an increase in the consumption of oxygen (Figure 5). Both endogenous and OA stimulated oxygen consumption was inhibited by KCN at a minimum final concentration of 3.8 mM (Figure 5). The endogenous oxygen consumption rate was (0.33 ± 0.04) μlO_2/min/mg dry weight (D.W) and upon addition of the OA oxygen consumption increased to (0.80 ± 0.05) μlO_2/min/mg D.W (QO_2 = 26). Addition of KCN

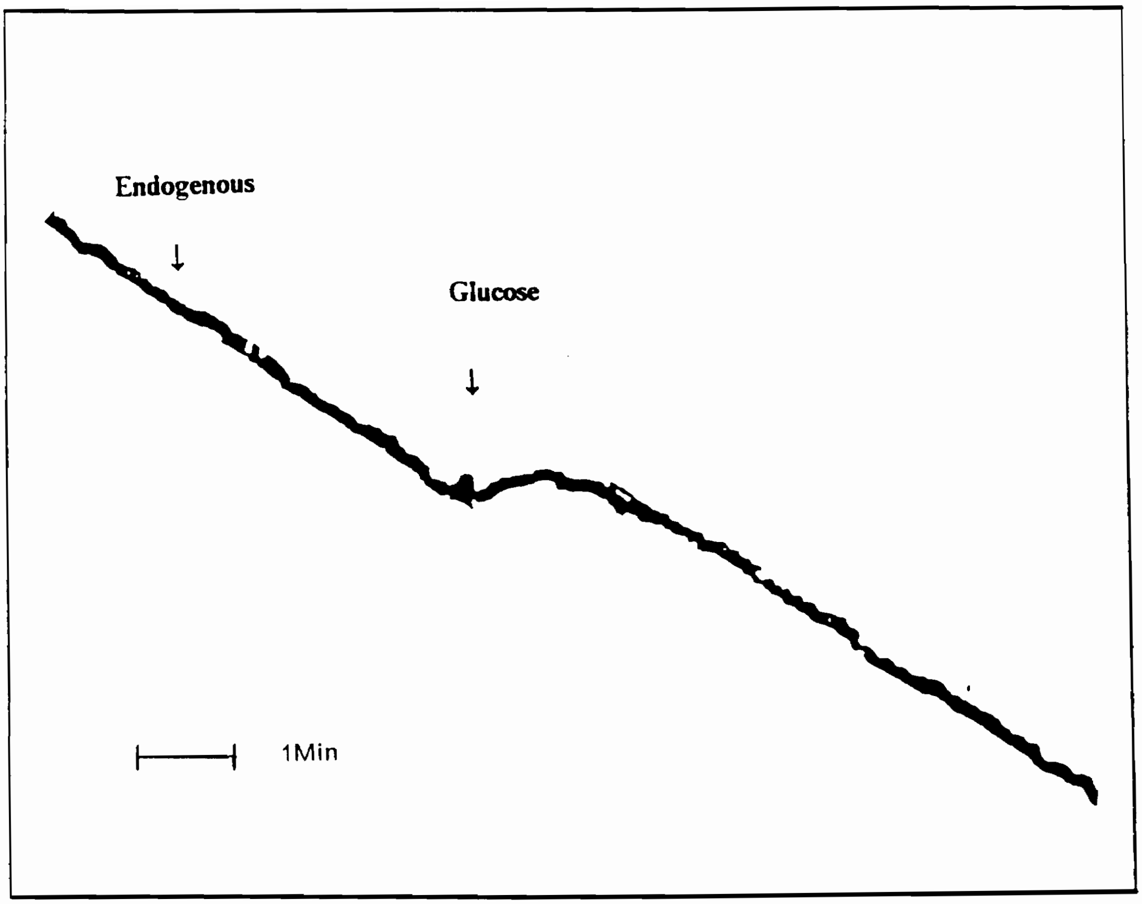

Figure 3. Time course of oxygen consumption in the presence of glucose in

M. intracellulare strain LR163.

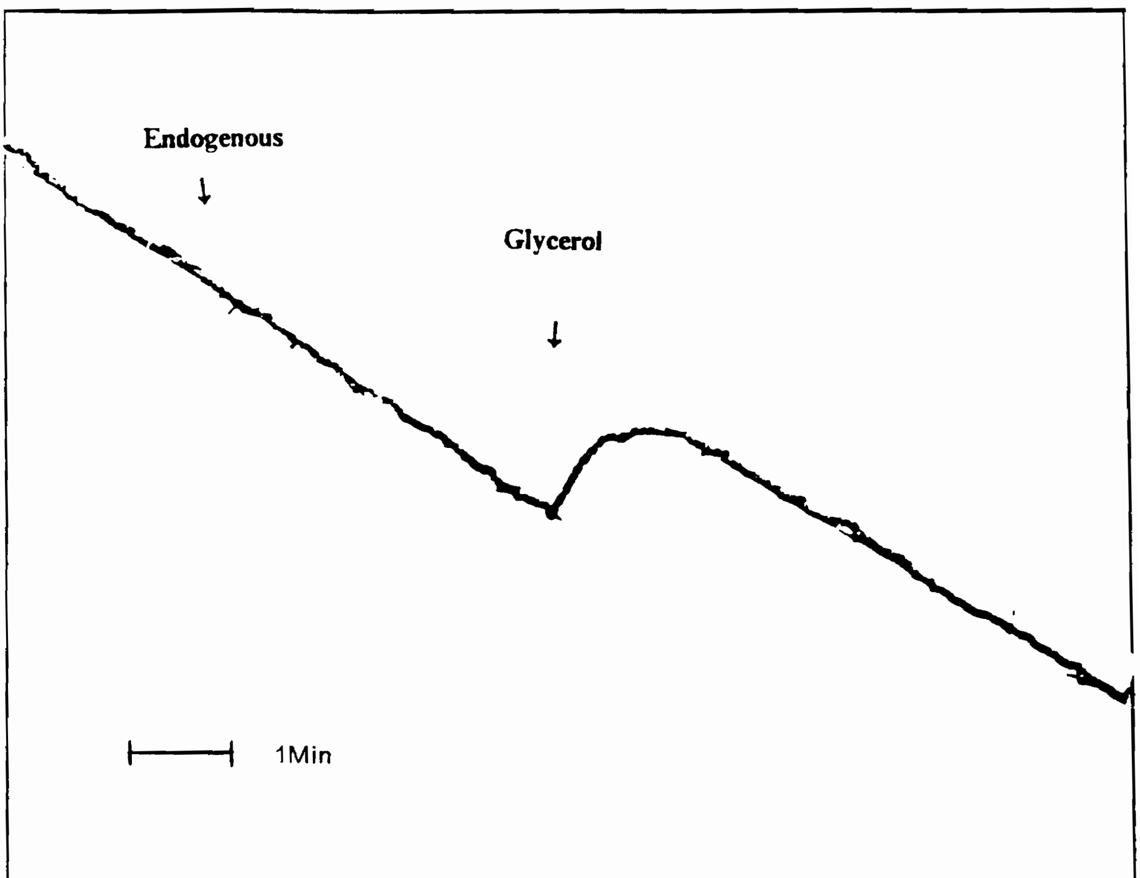

Figure 4. Time course of oxygen consumption in the presence of glycerol in *M. intracellulare* strain LR163.

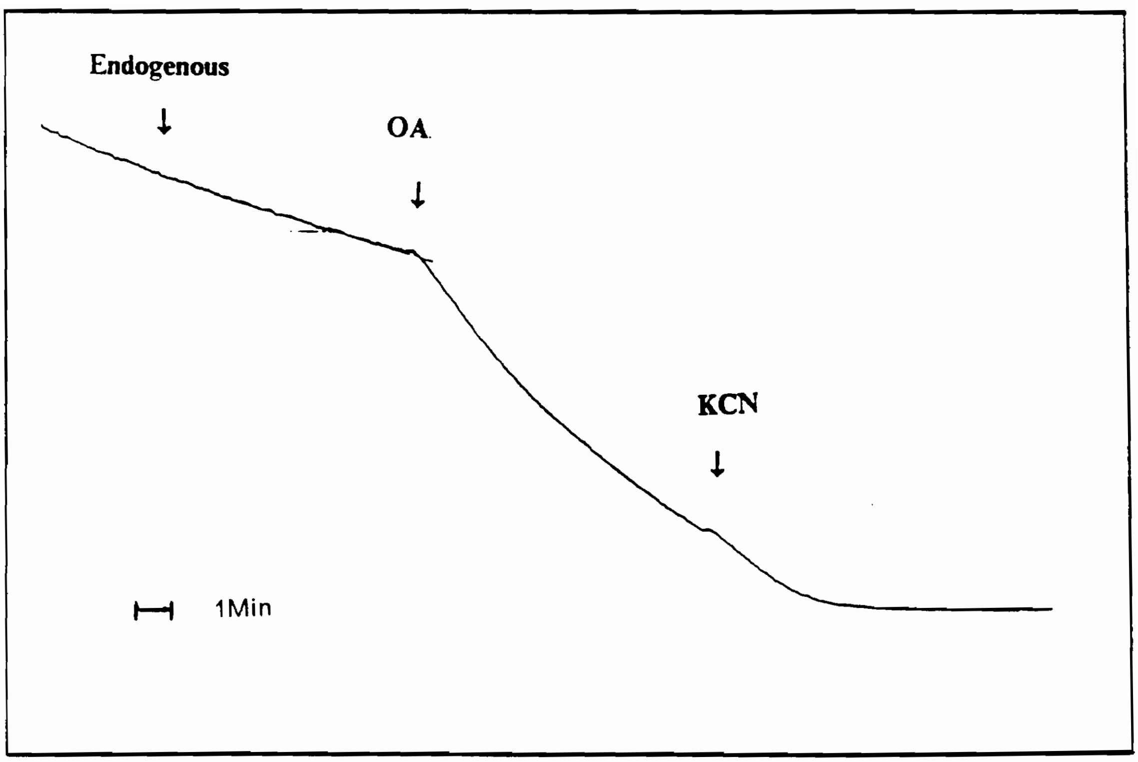

Figure 5. Time course of oxygen consumption in the presence of OA and OA and 3.8mM KCN in *M. intracellulare* strain LR163.

(3.8 mM) decreased this rate to (0.11 ± 0.00) $\mu l O_2$/min/mg dry weight (D.W) ($QO_2 < 0$) (Table 3).

CLOFAZIMINE-INDUCED OXYGEN CONSUMPTION

Clofazimine induced oxygen consumption by *M. intracellulare* strain LR163. The rate of clofazimine-induced oxygen consumption was dose-dependant between the range of 23.5 and 2.9 μg clofazimine/ml (Table 3, Figures 6-10) and was directly proportional to the amount of clofazimine added. There was no significant increase in the rate of oxygen consumption at concentrations below 2.9 μg clofazimine/ml (Figures 9 and 10).

Clofazimine-induced oxygen consumption was not inhibited by the addition of KCN (final concentration 3.8 mM). In fact, clofazimine-induced oxygen consumption was higher in the presence of KCN (Table 3, Figure 11). Above 11.8 μg clofazimine/ml, KCN did not stimulate oxygen consumption and at concentrations below 2.9 μg clofazimine/ml it led to an elevation in the amount of oxygen consumed above the endogenous level observed (Table 3, Figure 11).

Storage of the cell suspension at 4°C for four days did not significantly reduce ($0.05 < P < 0.10$) the endogenous respiration rate of the cells. The endogenous rates of the cell suspensions were determined daily. Starvation of the cells by incubation of the cell suspension at 37°C led to a slight decrease in the endogenous respiration ($< 0.33 \pm 0.04$ $\mu l O_2$/min/mg dry weight).

EFFECT OF GROWTH OF CELLS IN CLOFAZIMINE ON CATALASE ACTIVITY

Cells of *M. intracellulare* strain LR163 grown in the presence of 0.025 μg clofazimine/ml in MGE medium had 20% more catalase activity. By contrast, cells grown in the presence of 0.060 μg clofazimine/ml had 35% less catalase activity (Table 4). Measures were performed in triplicate on duplicate cultures.

EFFECT OF GROWTH OF CELLS IN CLOFAZIMINE ON SUPEROXIDE DISMUTASE ACTIVITY

A concentration of 0.133 μg superoxide dismutase/ml inhibited the PMS-induced rate of superoxide formation by 50% defining one unit of activity. This value was used to calculate the amount of SOD (μg/ml) from the units of SOD activity present in each extract. As seen in Table 5, SOD activity was 20% lower in cells grown in MGE medium containing 0.06 μg clofazimine/ml.

SYNERGISM

Clofazimine did not show synergism with streptomycin (FIC > 0.5) and was mildly synergistic in its mode of action with rifampicin (FIC = 0.4) in *M. intracellulare* strain LR163

Table 3. Oxygen consumption in *M. intracellulare* strain LR163

Treatment	O_2 consumed µl/min/mg D.W.	$Q_{O2.}$[a]
Endogenous	0.33 ± 0.04	—
Endogenous + 3.8 mM KCN	< 0.33	< 0
Glucose	0.33 ± 0.03	< 0
Glycerol	0.33 ± 0.03	< 0
OA	0.80 ± 0.05	26.1 ± 4.7
OA + 3.8 mM KCN	0.11 ± 0.0	< 0
CLOFAZIMINE		
23.5 µg Clofazimine/ml	0.63 ± 0.09	17.3 ± 1.5
11.8 µg Clofazimine/ml	0.50 ± 0.01	8.7 ± 1.5
5.9 µg Clofazimine/ml	0.40 ± 0.01	4.2 ± 1.5
2.9 µg Clofazimine/ml	0.33 ± 0.00	< 0
1.5 µg Clofazimine/ml	0.31 ± 0.01	< 0
CLOFAZIMINE + 3.8mM KCN		
23.5 µg Clofazimine/ml	0.75 ± 0.01	24.4 ± 2.6
11.8 µg Clofazimine/ml	0.79 ± 0.04	25.5 ± 0.0
5.9 µg Clofazimine/ml	0.64 ± 0.00	18.8 ± 0.0
2.9 µg Clofazimine/ml	0.48 ± 0.03	8.7 ± 1.2
1.5 µg Clofazimine/ml	0.36 ± 0.04	2.7 ± 1.2

[a] $Q_{O2.}$ = (µl O_2 consumed after addition of substrates) - (endogenous µl O_2 consumed)(60 min)/(mg/ml dry weight)

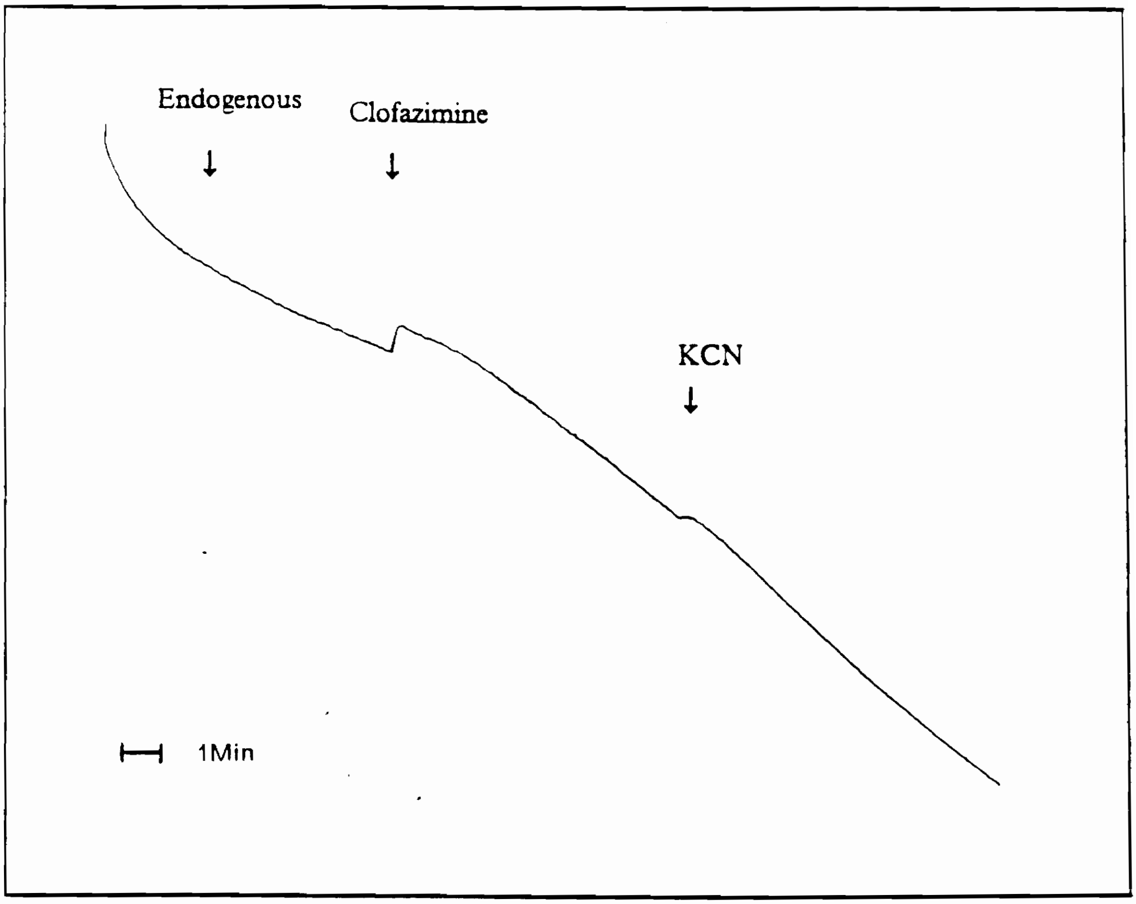

Figure 6. Time course of oxygen consumption in the presence of 23.5 μg clofazimine/ml and 23.5 μg clofazimine/ml and 3.8mM KCN in *M. intracellulare* strain LR163.

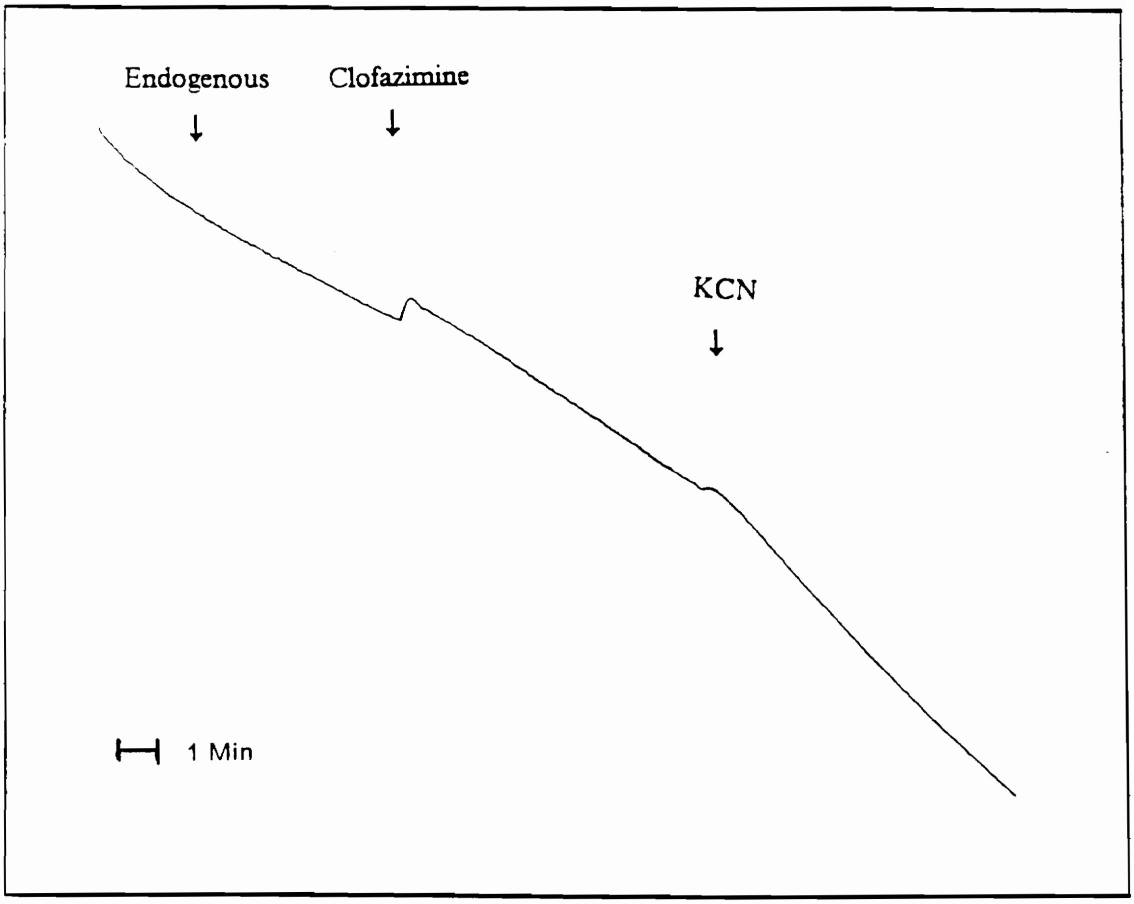

Figure 7. Time course of oxygen consumption in the presence of 11.8 μg clofazimine/ml and 11.8 μg clofazimine/ml and 3.8mM KCN in *M. intracellulare* strain LR163.

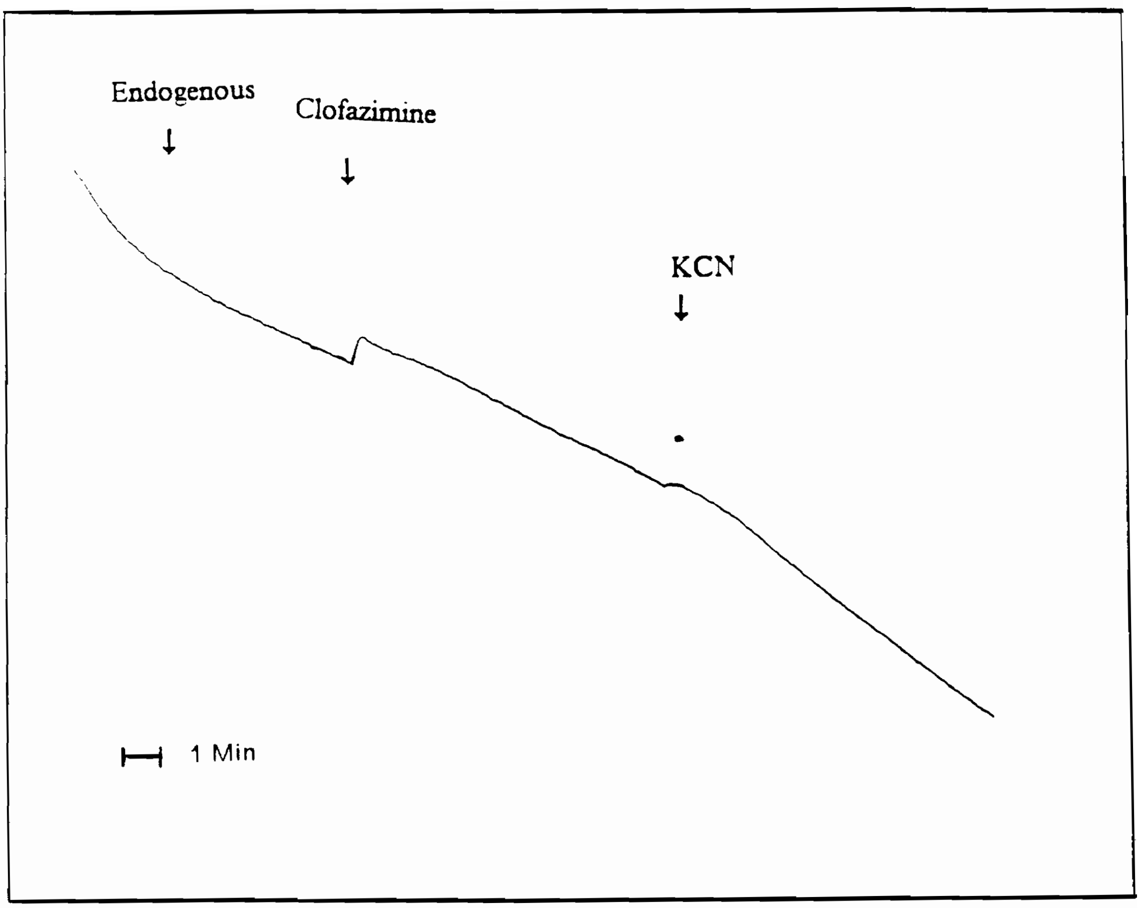

Figure 8. Time course of oxygen consumption in the presence of 5.9 μg clofazimine/ml and 5.9 μg clofazimine/ml and 3.8mM KCN in *M. intracellulare* strain LR163.

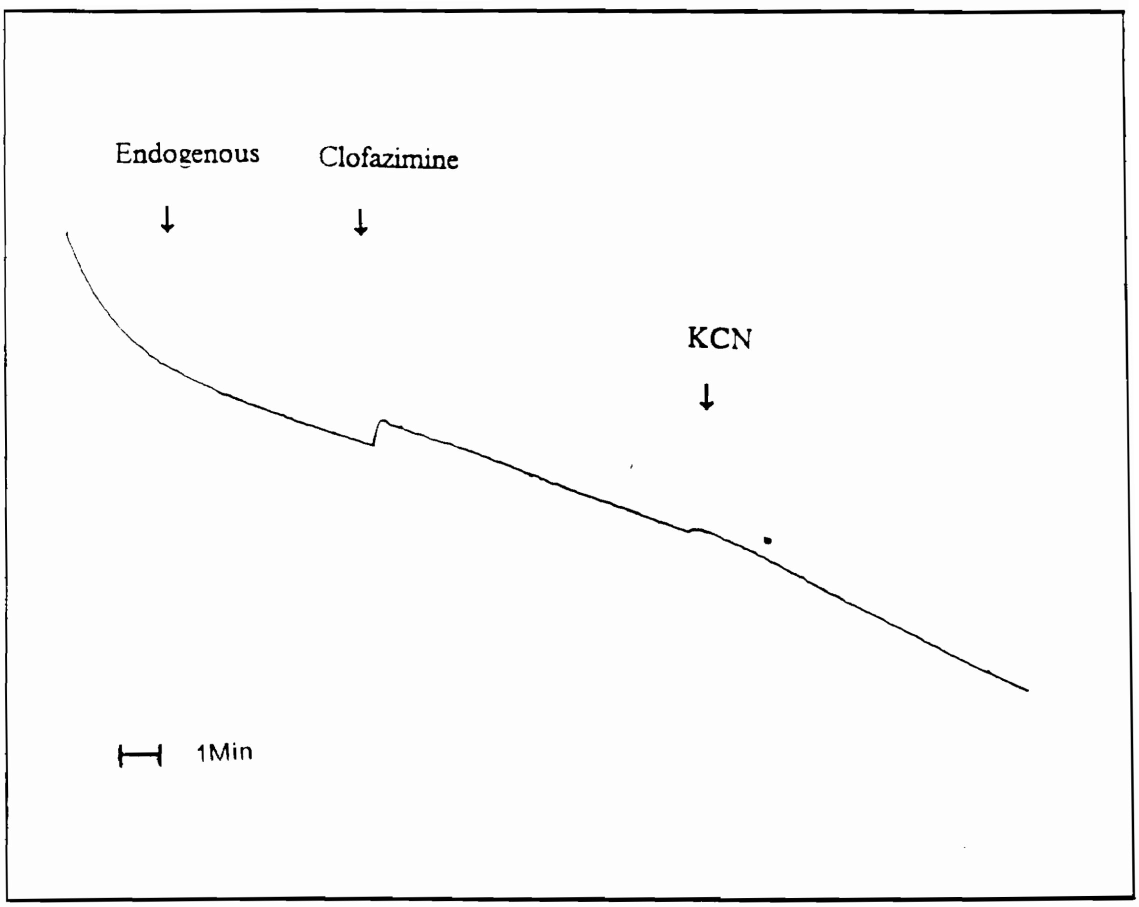

Figure 9. Time course of oxygen consumption in the presence of 2.9 μg clofazimine/ml and 2.9 μg clofazimine/ml and 3.8mM KCN in *M. intracellulare* strain LR163.

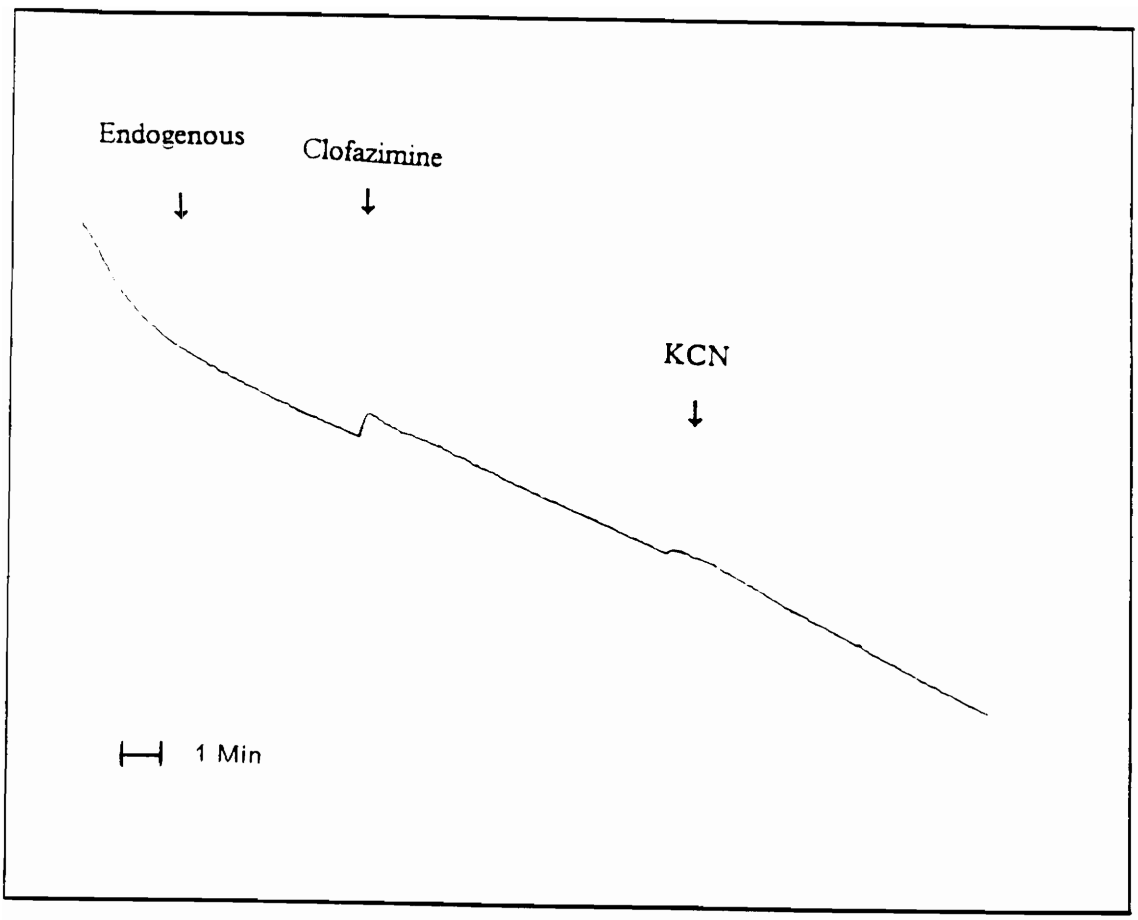

Figure 10. Time course of oxygen consumption in the presence of 1.5 μg clofazimine/ml and 1.5 μg clofazimine/ml and 3.8mM KCN in *M. intracellulare* strain LR163.

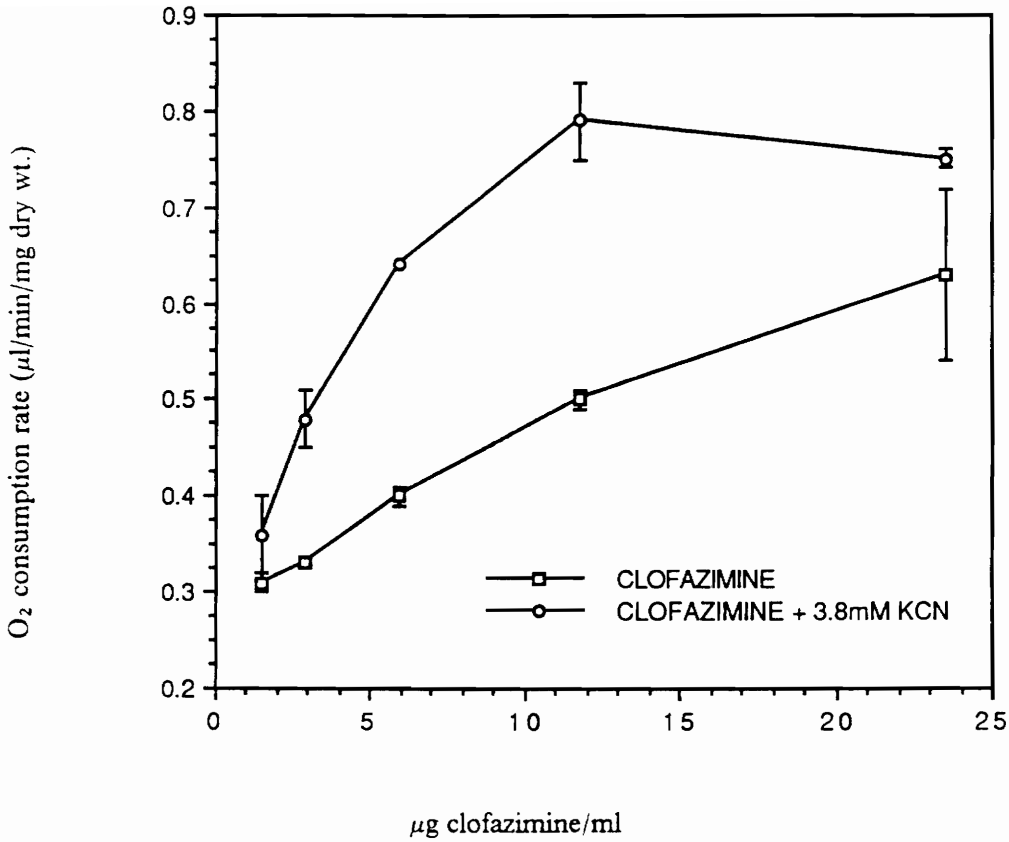

Figure 11. Clofazimine-stimulated oxygen consumption by *M. intracellulare* strain LR163 in the presence and absence of 3.8 mM KCN.

Table 4. Catalase activity of cells of *M. intracellulare* strain LR163 grown in the presence and absence of clofazimine

Clofazimine [a] μg/ml	Protein [b] concentration	Catalase [c] activity
Experiment 1		
0.000	0.50 ± 0.001	34 ± 3
0.025	0.51 ± 0.040	42 ± 6
Experiment 2		
0.000	0.36 ± 0.003	46 ± 5
0.060	0.30 ± 0.000	29 ± 1

[a] Clofazimine concentration in MGE broth

[b] Protein content of extract expressed as mg/ml

[c] Catalase activity expressed as units/mg protein

(Table 6). The FIC values were calculated with respect to clofazimine only. The MIC values obtained for each of the drug and in combination are listed in Table 6. Less than 99% killing occurred at the highest concentration of each of the antibiotics tested. Thus the MBC and FBC could not be determined.

ISOLATION OF CLOFAZIMINE-RESISTANT MUTANTS

No colonies were observed on MGE agar containing 10 μg clofazimine/ml. A total of 14 putative spontaneous clofazimine-resistant mutants were picked from MGE agar containing 1.0 μg clofazimine/ml. Only 1 of these isolates grew on MGE agar containing 1.0 μg clofazimine/ml upon restreaking for purification. However, this isolate showed poor growth on the selective medium after growth in MGE broth lacking clofazimine.

In the presence of ethyl methane sulfonate, a 3mm zone of clearing was seen around the mutagen which was followed by confluent growth of cells with a negligible change in colony number. Only one isolate was seen at the periphery of this zone. This putative mutant isolate showed 3 different colony types when restreaked on MGE agar containing 1.0 μg clofazimine/ml. This isolate grew on 1.0 μg clofazimine/ml after growth in non-selective MGE broth.

Around the NTG crystals, a large zone of clearing was seen with numerous colonies at its periphery. These colonies also showed different types upon streaking. Thirty-seven representative putative mutants were picked and streaked on MGE agar containing 1.0 μg clofazimine/ml. Sixteen of these 37 isolates showed growth and often showed mixed colony

Table 5. Superoxide dismutase activity of cells of *M. intracellulare* strain LR163 grown in the presence and absence of clofazimine

Clofazimine [a] μg/ml	Protein [b] mg/ml	SOD [c] units/mg
0.000	0.29 ± 0.010	4.3 ± 0.1
0.060	0.33 ± 0.005	3.4 ± 0.2

[a] Concentration of clofazimine in medium

[b] Protein content of extract expressed as mg/ml

[c] SOD activity expressed as units/mg protein

Table 6. Synergism between clofazimine and streptomycin and rifampicin in *M. intracellulare* strain LR163.

Antibiotic(s)	MIC	FIC	MBC	FBC
Clofazimine (Cf)	0.5	N.A.	> 1.0	N.A.
Streptomycin (Sm)	1.0	N.A.	> 1.0	N.A.
Rifampicin (Rf)	0.05	N.A.	> 0.05	N.A
Cf + Sm	0.5 Cf + 0.5 Sm	> 0.5	0.5 Cf > 0.5 Sm	> 0.5
Cf + Rf	0.2 Cf + 0.02 Rf	0.4	1.0 Cf > 0.1 Sm	> 0.5

types. All of the 16 isolates grew on the 1.0 μg clofazimine/ml after growth in the non-selective MGE broth.

DISCUSSION

MEASUREMENT OF CLOFAZIMINE IN MEDIUM

The standard curve for the measurement of the amount of clofazimine in medium showed a linear relation to absorbance above concentrations of 0.31 μg clofazimine/ml. Values are means of duplicate measurements of two separate dilution series. The loss of linearity below the concentrations of 0.31 μg clofazimine/ml could be the result of interference from medium components. The standard curve obtained can be used in the estimation of the amount of clofazimine in medium.

PIGMENTATION

M. avium strain 2812P was chosen to measure the effect of growth in clofazimine on carotenoid pigment production because few *M. avium* complex strains are pigmented and pigmentation was most evident in this strain (Stormer and Falkinham, 1989). An increase in the amount of pigment was seen in cells grown at all concentrations of clofazimine. This result supported the hypothesis that pigmentation in *M. avium* may protect cells against the toxicity of clofazimine. Pigments have been shown to quench superoxide radicals (Krinsky, 1979; Burton and Ingold, 1984).

The increase in the amount of pigment produced was not linear in relation to the amount of clofazimine in the medium (Figure 2). At 0.015 μg clofazimine/ml the amount of pigment produced was much higher than at any other concentration and the growth of cells was considerably inhibited (Table 2). One possible explanation for this observation may be that this concentration of antibiotic was below the threshold level required for the induction of enzymes such as catalase or superoxide dismutase. Thus at 0.015 μg clofazimine/ml, pigmentation was the primary defensive response. Clearly, pigment alone was not enough to protect the cells because growth was inhibited. The amount of pigment in cells grown in the presence of 0.06 μg clofazimine/ml was not significantly higher than that of cells grown without clofazimine. Further, cell growth was not inhibited at that concentration. Possibly, defense mechanisms, other than pigment induction, were effective in protecting the cells at this concentration. Dry weight values used in determining the amount of pigment present in the extracts were that of the culture and not of the cell suspensions used in extraction. A loss in the amount of cells may have occured in the making of the cell suspension. This loss would affect the calculations of the amount of pigment present in the extracts. This problem is probably minimal but future experiments need to measure the dry weights of the cell suspensions to give a true picture.

These results need to be confirmed by repeating the experiment under the same conditions and measuring catalase and superoxide dismutase activities with pigmentation. In addition, a wider range of clofazimine concentrations can be used. This phenomenon may be unique to strain 2812P, therefore other *M. avium* strains need to be studied.

CLOFAZIMINE-INDUCED OXYGEN CONSUMPTION

Clofazimine induced cyanide-resistant oxygen consumption by *M. intracellulare* strain LR163. The amount of oxygen consumed was dose-dependant. This observation agreed with the hypothesis that clofazimine is antibiotic by virtue of its ability to generate superoxide. The mechanism by which clofazimine achieves this is unknown but may be similar to the reduction of paraquat (a phenazine pigment) in *Escherichia coli* (Hassan and Fridovich, 1979). The reason for the increase in the clofazimine-induced oxygen consumption upon addition of KCN is not known. A similar phenomenon was observed in the paraquat-induced cyanide-resistant oxygen consumption by *Escherichia coli* (Hassan and Fridovich, 1979). The concentrations of clofazimine required to observe the clofazimine-induced cyanide-resistant oxygen consumption were in the range of 5 to 25 times higher than the MIC value. However this response is likely significant because the MIC values were measured after an incubation period of 7 days at 37°C, while the effect of clofazimine on oxygen consumption was measured at 37°C over a ten minute period. Absence of glucose or glycerol-stimulated oxygen consumption may be due to the impermeability of these substrates in the cells or that the intracellular concentrations of the carbohydrates was equal to or higher than the extraneous amount added leading to no change in the rate of oxygen consumption (Tepper, 1968).

EFFECT OF GROWTH OF CELLS IN CLOFAZIMINE ON CATALASE ACTIVITY

M. intracellulare strain LR163 catalase activity showed a 20% increase when the cells were grown in the presence of 0.025 μg clofazimine/ml. However in the presence of 0.060 μg clofazimine/ml there was a 35% decrease in catalase production. This decrease in the activity of catalase with an increase in the concentration of clofazimine was unexpected. A possible explanation for these results is that at a concentration of 0.025 μg clofazimine/ml, the increase in catalase activity was effective in protecting the cells. Reduction in catalase activity at 0.06 μg clofazimine/ml could be due to the induction or activation of enzymes other than catalase. The amount of superoxide and hydrogen peroxide produced at this clofazimine concentration may overcome the ability of catalase to protect cells. The amount of protein in the extract indicated no inhibition in the growth of cells. This suggested that they were capable of surviving in the presence of 0.06 μg clofazimine/ml without inducing (activating) catalase. The amount of catalase units measured in the absence of clofazimine showed a day to day variation (i.e. between experiment 1 and 2). However the repeats within each day, did not show a wide variation. Extracts used for measuring catalse activity contained cell membranes. However, there was no effect of membranes on the catalase activity as shown by George and Falkinham (1989).

These results suggest that at higher concentrations of clofazimine, *M. intracellulare* could use a defense mechanism other than higher catalase activity. This could be confirmed by assaying for catalase at concentrations higher than 0.06 μg clofazimine/ml. Increased superoxide dismutase activity without increase in the catalase activity was another possible defense mechanism that cells could use against superoxide radicals.

EFFECT OF GROWTH OF CELLS IN CLOFAZIMINE ON SUPEROXIDE DISMUTASE

Extracts of cells of *M. intracellulare* strain LR163 had 20% less superoxide dismutase activity when grown in the presence of 0.06 μg clofazimine/ml than grown without clofazimine.

In addition there was no inhibition of the growth of cells. The cells survived in the presence of 0.06 μg clofazimine/ml without activating or inducing superoxide dismutase. This result was not expected. Assaying for the activity of superoxide dismutase of cells grown in higher concentrations of the antibiotic would confirm if this enzyme played an important role in the defense of the cells against the antibiotic.

As previously discussed, catalase activity and the amount of pigment also showed a decrease in the presence of 0.06 μg clofazimine/ml. Thus, neither superoxide dismutase or catalase were responsible for the mechanism of defense against the superoxide radicals generated by clofazimine at this concentration. Extracts used for measuring superoxide dismutase activity contained cell membranes and the measurement of activity was probably effected by the presence of membranes (George and Falkinham, 1989). Therefore these experiments need to be repeated in the absence of membranes and with a wider range of clofazimine concentration to confirm the observations seen here.

SYNERGISM

In the presence of clofazimine, the MIC values of *M. intracellulare* strain LR163 were not lowered upon addition of streptomycin (inhibitor of protein synthesis). The MIC value for streptomycin was relatively high indicating that the cells were resistant to streptomycin. This could be due to a permeability barrier or a mechanism by which streptomycin was inactivated by the cells. Thus, streptomycin was not an effective drug and this could have resulted in the lack of synergism between clofazimine and streptomycin.

Clofazimine was mildly synergistic with rifampicin (inhibitor of RNA synthesis). The MIC of the drugs when used in combination were about half the concentration of the MIC of each drug when present by itself. The FIC value (Yajko, et., al., 1988) indicated that the drugs were mildly synergistic. This finding agreed with the hypothesis that clofazimine induced enzymes for protection against the drug. The lack of strong synergism between clofazimine and rifampicin could have been due to employment of low antibiotic concentrations. The experiments were not repeated using higher antibiotic concentrations, because higher concentrations than those used here are unattainable (toxic) in human serum.

ISOLATION OF CLOFAZIMINE-RESISTANT MUTANTS

The isolation of clofazimine-resistant mutants provides an opportunity to identify mutant phenotypes that lead to resistance. Almost all mutants isolated were those induced by NTG, none spontaneously or by ethyl methane sulfonate. At a concentration of 10 μg clofazimine/ml there were no spontaneous or mutagen-induced MAIS survivors. This concentration was 10 times higher than the MIC value determined and probably killed all the cells.

SUMMARY

The presence of clofazimine in growth medium resulted in higher carotenoid pigmentation in *M. avium* cells. Carotenoid pigments have been shown to quench superoxide radicals. (Krinsky, 1989; Burton and Ingold, 1984) supporting the hypothesis that pigmentation, possibly protected cells against superoxide. For reasons not yet known, the amount of pigment produced was not proportional to the amount of clofazimine present in growth medium. This trait may be unique to *M. avium* strain 2812P and further study with other strains is required. Clofazimine caused the generation of superoxide in *M. intracellulare* strain LR163 represented by cyanide-resistant oxygen consumption. The amount of oxygen consumed was dependant upon the clofazimine concentration. This supported the hypothesis that clofazimine was antibiotic via its ability to generate toxic oxygen metabolites, a trait observed in other phenazine pigments. Higher catalase activity was found in extracts of cells grown in the presence of a low concentration of clofazimine. At a higher concentration, the amount of catalase and superoxide dismutase activities were lower than the basal level. This finding did not agree with the hypothesis. Further study on the effect of higher concentrations of the antibiotic would confirm if these enzymes were indeed involved in the defense mechanism by the cells against clofazimine. In the case of superoxide dismutase activity, it is likely that the presence of the membrane fraction strongly influenced the results. At this point the reason for the drop in the activities (i.e. lower than basal level) is not known.

One possibile explanation could be that in the presence of a certain concentration of clofazimine the cells may induce cell-surface modifications leading to permeability barriers. The MIC of clofazimine was 1.0 μg/ml of the drug. The concentrations of the drugs used in medium for inducing (activating) superoxide dismutase, catalase, and pigmentation was less that 10% of the MIC value. It could be possible that the lack of activation of the enzymes seen was a result of the low concentration of the drug in the medium.

Clofazimine was mildly synergistic with rifampicin. This result supported the hypothesis that the defense mechanism of *M. intracellulare* to clofazimine was enzymatic.

Because strain LR163 was not very susceptible to streptomycin, there was no synergism observed between clofazimine and streptomycin.

The characterization of the clofazimine-resistant mutants may resolve some of the issues above. Strains resistant to clofazimine have been successful in surviving the harmful effects of the drug thus they provide a direct approach towards determining the mode of action of clofazimine in cells. Resistance of the mutants to clofazimine may be due to permeabilty barriers, increased pigmentation, higher catalase or superoxide dismutase activities or some other intracellular defense mechanism.

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APPENDIX

EVIDENCE THAT THE ABILITY OF MAIS TO GROW AT 43°C IS PLASMID-ENCODED

INTRODUCTION AND REVIEW OF LITERATURE

Mycobacterium avium, *Mycobacterium intracellulare* and *Mycobacterium scrofulaceum* (called the MAIS group) have been isolated from water (Falkinham et al., 1980), aerosols (Wendt et al., 1980) and soil (Brooks et al., 1984). The frequency of MAIS recovery (Falkinham et al., 1980) and MAIS numbers (Brooks et al., 1984) are higher in southeastern than in northeastern United States soil and water. That distribution coincides with the higher frequency of recovery of these organisms from patient samples sent to state tuberculosis diagnostic laboratories (Good, 1980; Good and Snider, 1982) and to the higher frequency of blood samples containing anti-MAIS antibodies (Edwards et al., 1969; Edwards, 1970).

MAIS organisms are slow growing opportunistic pathogens and *M. avium* and *M. intracellulare* cause pulmonary infections (mycobacteriosis) in humans (Wolinsky, 1979). Recently it has been shown that 25% of patients with acquired immunodeficiency syndrome (AIDS) (AIDS; Zakowski, et al., Winter, et al., 1985; Wong, et al., 1985) or Kaposi's sarcoma (Fainstein, et al., 1982) or under immunosuppressive drug treatment (Simpson, et al., 1982) have disseminated MAIS infections (Blaser and Cohn, 1986). In the absence of person-to-person transfer (Wolinsky, 1979), the apparent source of human MAIS infection is environmental. This is supported by the fact that MAIS isolates from patients with mycobacteriosis and those from aerosols share common characteristics (Meissner and Falkinham, 1986; Fry et al., 1986).

Growth at 43°C (Fry et al., 1986) and the presence of plasmid DNA (Meissner and Falkinham, 1986) are two of the characteristics shared by clinical and aerosol isolates. The ability to grow at 43°C was found to correlate with the presence of plasmids suggesting that the ability to grow at 43°C was plasmid-encoded (Fry et al., 1986). *M. avium* strain LR25 grows at 43°C while its plasmid-free derivative (strain LR163) does not. This suggests that MAIS plasmids may encode for the ability to grow at 43°C. Further, strain LR25 was virulent in the beige mouse, while strain LR163 was avirulent (Gangadharam, et al., 1988) *M. avium* strain LR163 lacks a heat-stable catalase active at 43°C which is present in strain LR25. (Pethel and Falkinham,1989).

The hypothesis was that MAIS plasmids may encode for a catalase activity which functions in pathogenesis. The objective of the study here was to identify the plasmid encoding for the ability to grow at 43°C.

Two approaches were used to confim the hypothesis that plasmid-encoded genes permit strain LR25 to grow at 43°C. In the first approach, *M. avium* strain LR25 was grown in a medium containing agents which would enhance the loss of plasmids (i.e. curing). Curing agents preferentially inhibit plasmid DNA replication and segregation. Significant lower viable counts of strain LR25 on media at 43°C, compared with 37°C which do not occur in an untreated culture might indicate plasmid loss. In addition, colonies of strain LR163 are yellow, while those of strain LR25 are white when grown on medium containing pyruvate (MGEP agar). Presence of yellow-pigmented colonies in cultures of strain LR25 exposed to curing agents might indicate appearance of strain LR163-type, cured cells. This trait could possibily be due to the loss of plasmid(s). Any putative cured clones obtained would be grown and plasmids separated by agarose gel electrophoresis as described by Jucker and Falkinham,

(1990) to identify strains lacking one or more plasmids. Based on the ability of a cured clone to grow at 43°C and the plasmid content of the clone, it might be possible to assign the gene(s) for growth at 43°C to one of the three plasmids.

A second approach for determining which plasmid contained the genetic determinant for growth at 43°C, involved transfer of individual plasmids from strain LR25 into the plasmid-free strain LR163 by electroporation. Electroporation introduces DNA into cells by means of electrical pulses; the electrical pulse makes cells permeable to plasmids (Snapper, et al., 1988). Successfully transfected strains will be characterized for growth at 43°C. Plasmid analysis of the electroporated clone capable of growth at 43°C will confirm the assignment of this trait to one of the plasmids. Electroporation of *Mycobacteria smegmatis* strain $mc^2 6$ lacking plasmids with a recombinant plasmid pYUB12 carrying kanamycin-resistance genes (Snapper, et al., 1988), was used as a model to determine parameters of electroporation for use in electroporation of strain LR163 with strain LR25 plasmids. Selection of kanamycin-resistant transformants was done by growth of the electroporated cells on media containing 10 μg kanamycin/ml. Cells lacking pYUB12 were incapable of growth on this selective medium.

MATERIALS AND METHODS

BACTERIAL STRAINS

PLASMID CURING. *Mycobacterium avium* strain LR25 and *Mycobacterium intracellulare* strain LR163 (Crawford, et al., 1985) were used in this study.

ELECTROPORATION. *Mycobacterium intracellulare* strain LR163 (Crawford, et al., 1985), *Escherichia coli* strain HB101 transformed with pYUB12 (Snapper, et al.,), and *Mycobacterium smegmatis* strain $mc^2 6$ (Snapper, et al.,) were used in this study.

MEDIA

PLASMID CURING

The mycobacterial cultures were grown in Middlebrook 7H9 broth medium (BBL Microbiology Systems, Cockeysville, MD.) containing 0.2% (w/v) glucose and 0.05% (v/v) Tween 80 (MGT broth). Cultures for plasmid analysis were grown as described by Jucker and Falkinham (1990) in M7H9 broth containing 0.5% (v/v) glycerol and 10% (v/v) oleic acid albumin (OA) (Sigma Chemical Co. St. Louis, MO). OA was prepared as described by Pethel and Falkinham (1989). Stock cultures were grown in 2 ml MGT broth to mid-log phase (10 days) at 37°C in screw-cap tubes (18x150 mm), vortexed daily for aeration and stored at 4°C.

The agar medium used was Middlebrook 7H10 (BBL, Microbiology Systems, Cockeysville, MD) containing 0.5% (vol/vol) glycerol and 10% (vol/vol) OA (MGE agar). MGE agar containing 0.66% (w/v) pyruvate (Sigma Chemical Co., St. Louis, MO) (MGEP agar) was used as well. Plates were sealed with Parafilm (American National Can, Greenwich, CT) and incubated at 37°C for 3 (MGE agar) and 5 weeks (MGEP agar) respectively. All water used in the preparation of media was glass-distilled.

Plate count agar (PCA, Difco Laboratories, Detroit, MI) was used to detect contaminants. Medium or culture was streaked (0.001 ml) and plates incubated at 37°C for 24 hours.

ELECTROPORATION

Mycobacterial cultures were grown in Middlebrook 7H9 broth medium (BBL Microbiology Systems, Cockeysville, MD) containing 0.5% (v/v) glucose and 10% (v/v) albumin dextrose complex (ADC) (Sigma Chemical Co., St. Louis, MO) (MGA broth). Cultures for plasmid analysis were grown as described by Jucker and Falkinham (1990) in M7H9 broth containing 0.5% (v/v) glycerol and 10% (v/v) ADC. Stock cultures of *Mycobacterium smegmatis* were grown in 2 ml MGA broth for 3 days (log phase) at 37°C in screw-cap tubes (18x150 mm) and vortexed daily for aeration and then stored at 4°C.

The agar medium used was Middlebrook 7H10 (BBL, Microbiology Systems, Cockeysville, MD) containing 0.5% (v/v) glycerol and 10% (v/v) ADC (MGA agar). Selective agar plate medium used for the isolation of transformants was MGA agar containing 10 μg kanamycin (Sigma Chemical Co., St. Louis, MO)/ml (MGAK agar). Plates were sealed with Parafilm (American National Can, Greenwich, CT) and incubated at 37°C for three to four days. All water used in the preparation of media was glass-distilled.

The *Escherichia coli* cultures were grown in Terrific Broth (Sambrook, et al., 1988) containing 10 μg kanamycin/ml. These cultures were incubated at 37°C for 16 to 18 hours for the purpose of isolating pYUB12 plasmid DNA. Agar medium used was that of Luria (Maniatis, et al., 1982) containing 10 μg kanamycin/ml. Plates were incubated at 37°C for 24 hours.

Plate count agar (PCA, Difco Laboratories, Detroit, MI) was used to detect contaminants. The medium was streaked with a 0.001 ml loop containing culture and incubated at 37°C for 24 hours.

ANTIBIOTICS AND REAGENTS

PLASMID CURING

Sodium dodecyl sulfate (SDS), acridine orange (AO), neutral acriflavin (NA), mitomycin A (MC), ethidium bromide (EB), novobiocin (NOV) and rifampicin (RIF) (all from Sigma Chemical Co., St. Louis, MO) were prepared by dissolving powders (w/v) in sterile distilled water and filter-sterilized before use. (rifampicin was dissolved in 10% methanol). All antibiotics solutions were foil-covered and transfers involving these solutions were performed in the dark.

PLASMID ANALYSIS

Ampicillin and D-cycloserine (Sigma Chemical Co., St. Louis, MO) were prepared by dissolving the powder (w/v) in water. Solutions were filter-sterilized before use.

ELECTROPORATION

Kanamycin solution was prepared by dissolving kanamycin sulfate powder (Sigma Chemical Co., St. Louis, MO) in water. The solution was filter-sterilized before use.

PLASMID CURING

Cultures were grown in 5 ml of MGT broth containing one of the curing agents, listed in Table 1. All tubes were foil-covered to protect the curing agents from light. Cultures for UV treatment were placed in the dark in a sterile petri dish and irradiated with 360 ergs/mm^2 (280 nm). Following irradiation, the cultures were transferred to the original tubes. Cell density of the inoculum was determined by dilution plating on MGE agar.

To determine if curing had occurred, the cultures from each of the thirteen treatments were streaked on two MGE agar plates in duplicate with a 0.001 ml loop after 0, 3, 7, and 10 days incubation in the presence of the curing agents. After 10 days of incubation the cultures were transferred to 4°C for storage. Each plate was sealed with Parafilm (American National Can, Greenwich, CT) and one incubated at 37°C, the other at 43°C for 15 days. Plates were observed for colonies and inhibition, expressed as fewer colonies at 37°C compared to the drug free control and curing, expressed as fewer colonies at 43°C compared to 37°C measured.

ISOLATION OF PUTATIVE CURED ISOLATES

Based on their inhibition of growth, treatments consisting of 0.005% (w/v) SDS/ml, 0.5 μg neutral acriflavin/ml and 10 μg novobiocin/ml to isolate cured derivatives of strain LR25 were attempted. Ten colonies from MGE plates at 37°C and 10 colonies from MGE plates at 43°C were picked from each treatment (i.e. 20 isolates for each representative treatment) and suspended in 2 ml MGT broth medium in 16 X 125mm screw cap tubes, cultures grown and plasmids characterized (above). Colonies were not restreaked before analysis and hence were not pure isolates.

MGE agar containing pyruvate was used to isolate yellow-pigmented colonies by plating strain LR25 cell suspensions from each treatment on MGEP agar plates. The plates were sealed with Parafilm (American National Can, Greenwich, CT) and incubated at 37°C for 5 weeks. The percentage of yellow colonies among white colony forming units (CFU) on the same MGEP plate was calculated to give an estimate of the frequency of putative-cured derivatives (if yellow pigment was indeed a true indicator of plasmid loss). Inhibition of growth, due to the various treatments was calculated by comparing the total number of CFU (yellow + white) in the presence of the treatment to the total number of CFU in the control (no treatment). Depending on the percentage of yellow colonies, 1 to 10 isolated yellow colonies from each treatment were picked and restreaked on MGEP agar to obtain a pure isolated colony For treatments showing a higher frequency of yellow colonies, up to 10 isolated colonies were picked. The plates were sealed with Parafilm and incubated at 37°C for 6 weeks. Isolated colonies were picked and suspended in 2 ml MGT broth medium in 16 X 125 mm screw cap tubes and incubated at 37°C for 15 days as inoculum for cultures for plasmid analysis.

PLASMID ANALYSIS

Procedure used for the analysis of plasmids was that of Jucker and Falkinham (1990).

ELECTROPORATION

Cultures for the the purpose of transformation by electroporation were grown in 100 ml MGE broth with continuous shaking at 100 rpm (Orbital Shaker Model 3590, Lab Line Instruments Inc. Melrose Park, IL) in 1000 ml, screw-cap flasks at 37°C for 6 days (log phase). Cells were harvested by centrifugation at 7,000 x g for 10 min at 4°C, washed once in distilled water by vortexing and pelleted by centrifugation at 7,000 x g for 10 min at 4°C. The cell pellet was resuspended in sterile distilled water. A calibrated loopful of the cell suspension was streaked on MGE agar and PCA to determine viability and the absence of contaminants, respectively. This suspension was stored at -70°C.

ELECTROPORATION IN *M. SMEGMATIS*

The recombinant plasmid pYUB12 coding for kanamycin-resistance was isolated from *Escherichia coli* strain HB101 carrying pYUB12 (Snapper, et al., 1988) by the procedure of Birnboim and Doly (1979). Initially, *Mycobacterium smegmatis* strain $mc^2 6$ was electroporated with pYUB12 using the same conditions as those used by Snapper, et al., (1988) without success. The experiment was repeated by : (1) increasing the number of pulses to two with a 10 sec delay between, (2) decreasing the volume of cells used by 60%, and (3) increasing the phenotypic lag period to 14 hours. A kananamycin-resistant transformant was obtained. The effect of the pulses on the viability of the cell was measured by plating cells before and after pulse(s) on MGE agar. Electroporated suspensions (100 μl) were plated on MGAK agar medium to select for kanamycin resistant transformants. Plates were sealed with Parafilm (American National Can, Greenwich, CT) and incubated at 37°C for 4 to 5 days. Putative transformant(s) were picked and streaked on MGAK plates for isolation. The transformant used in the studies here was named strain VT300.

ELECTROPORATION OF *M. AVIUM* STRAIN LR163

Transformation of *M. avium* strain LR163 with pYUB12 by electroporation was attempted using the same conditions as those used by Snapper, et al. (1988), with the exception that two pulses (instead of one pulse) were used with a 10 sec delay between pulses. The effect of increasing the phenotypic lag period on the transformation frequency of electroporation was determined by comparing the number of transformants obtained after incubation periods of 0.5, 3, 5, 11, and 27 hours. The occurrence of spontaneous mutants over this period was determined using the same conditions (but cells were not exposed to pYUB12 DNA). The possible occurrence of natural competence of strain LR163 was studied by adding pYUB12 DNA to the cell suspension followed by the addition of DNase (Sigma Chemical Co, St. Louis, MO) to the mixture at 0, 2, 4, 6, and 18 hours. This schedule permitted various lengths of time for DNA uptake and permitted calculation of the minimum time required for DNA uptake by the cells. Cells were plated on MGEK medium after 24 hours of incubation for phenotypic expression. Control for this experiment (i.e. occurence of spontaneous kanamycin-resistant mutants) consisted of cells in the absence of pYUB12 under the same conditions.

RESULTS

ISOLATION OF PUTATIVE CURED ISOLATES

In Table 1 and Table 2 are listed the percentage of strain LR25 colonies at 37°C and at 43°C (respectively) from cultures grown in the presence of the different curing agents compared to the control. Results from 7 days incubation were used to identify treatments that were most effective in inhibiting the growth of cells. Isolates growing at 37°C and 43°C from cultures containing 0.5μg neutral acriflavin/ml, 10 μg novobiocin/ml or 0.001% SDS were chosen for plasmid analysis. Of 20 colonies grown in 0.5μg neutral acriflavin/ml, 20 colonies grown in 10 μg novobiocin/ml and 10 colonies grown in 0.001% SDS, none showed altered plasmid profiles. The percentage of yellow pigmented colonies isolated from cultures exposed to curing agents as well as inhibition in growth are listed in Table 3. For reasons not known, colonies growing on pyruvate medium (MGAP agar) were not viable when transferred to MGT broth medium. Thus, isolates could not be tested for plasmid analysis.

Table A. Percent colony number of *M. avium* strain LR25 grown at 37°C in the presence of curing agents

Treatment	Sampling Date		
	Day 3	Day 7	Day 10
Control @ 37°C	100	100	100
Growth @ 43°C	243	166	128
UV @ day 4	88	16	5
0.005% SDS	64	169	26
0.001% SDS	112	131	70
1.5 μg AO/ml	195	271	109
0.5 μg NA/ml	60	17	5
0.1 μg NA/ml	188	128	122
20 μg MC/ml	1	< 1	< 1
10 μg MC/ml	6	< 1	< 1
0.1 μg EB/ml	220	185	36
10 μg Nov/ml	56	15	2
0.1 μg Rif/ml	13	< 1	0

Table B. Percent colony number ***M. avium*** strain LR25 grown at 43°C in the presence of curing agents

Treatment	Sampling Date		
	Day 3	Day 7	Day 10
Control @ 37°C	167	88	45
Growth @ 43°C	39	128	85
UV @ day 4	63	119	348
0.005% SDS	205	25	309
0.001% SDS	45	40	80
1.5 μg AO/ml	113	79	77
0.5 μg NA/ml	67	29	129
0.1 μg NA/ml	66	92	42
20 μg MC/ml	22	18	0
10 μg MC/ml	25	8	20
0.1 μg EB/ml	79	129	83
10 μg Nov/ml	36	23	0
0.1 μg Rif/ml	52	0	0

Table C. Effect of survival of cells and percent yellow pigmented cells of cultures of *M. avium* strain LR25 after growth in the presence of curing agents

Treatment	Percent Survival	Percent of Yellow CFU
Control @ 37°C	100	$< 2.6 \times 10^{-6}$
Growth @ 43°C	30	0.04
UV @ day 4	42	0.01
0.005% SDS	5	0.30
0.001% SDS	95	0.51
1.5 μg AO/ml	133	$< 2.6 \times 10^{-6}$
0.5 μg NA/ml	166	20.00
0.1 μg NA/ml	134	0.30
20 μg MC/ml	0	0
10 μg MC/ml	0.001	0.2
0.1 μg EB/ml	166	$< 1.6 \times 10^{-6}$
10 μg Nov/ml	0.001	0.25
0.1 μg Rif/ml	0	0

ELECTROPORATION

ELECTROPORATION OF *M. SMEGMATIS*

No kanamycin-resistant mutants of *M. smegmatis* mc^2 were recovered by electroporation with pYUB12 using the same conditions as those used by Snapper, et al., (1988). However, exposing the cells to 2 pulses (with a 10 sec delay), reducing volume of cell suspension by 60% and increasing the phenotypic lag period to 14 hours resulted in kanamycin-resistant transformants. (1 to 5 transformants/cell/μg pYUB12). There was no effect of the pulses on the viability of the cells. Infact, a slight increase in the number of colony forming units was often seen after the pulses. The transformant used in the study here was named strain VT300.

Proof that strain VT300 was a tranformant containing pYUB12 was provided by the observation of a single plasmid band of size 11.3 Kb upon plasmid analysis. Kanamycin-sensitive cured segregants were isolated by growing the transformant (VT300) in the absence of kanamycin. Cells from this suspension were plated on non-selective MGA plates and then transferred to selective MGAK plates by replica plating. The kanamycin-sensitive cured derivative used for the study here was named strain VT307 and lacked the 11.3 Kb plasmid seen in strain VT300. Transformation frequency of strain VT307 electroporated with pYUB12 was in the range of 1 x $10^5/\mu$g pYUB12 DNA using the same conditions as those of Snapper, et al., (1988). As observed by Snapper, et al. (1988), *M. smegmatis* cells transformed by pYUB12 are transformation-proficient mutants.

The effect of increasing the phenotypic lag period on the transformation frequency of *M. smegmatis* strain VT307 showed that there was no significant change if kanamycin-resistance was enumerated at 0.5, 3, 5, 11, and 27 hours following electroporation. No spontaneous mutants were detected in this period. Strain VT307 did not show natural competence in the presence of pYUB12 leading to kanamycin resistance.

ELECTROPORATION OF *M. INTRACELLULARE* STRAIN LR163.

Electroporation of strain LR163 with pYUB12 using the same conditions as those of Snapper, et al. (1988), did not lead to any kanamycin-resistant transformants. No cell death was observed after the pulses. The frequency of spontaneous kanamycin-resistant mutants was was in the range of 1 x 10^{-7}/cell. Strain LR163 did not show natural competence in the presence of pYUB12.

DISCUSSION

PLASMID CURING

The failure to detect plasmid loss in isolates of *M. avium* strain LR25 following exposure to curing agents could be due to the small number of isolates analysed. Growth of cells in the presence of 0.5 μg neutral acriflavin/ml, 10 μg novobiocin/ml, or 0.001% SDS did inhibit the growth of cells. Future studies involving plasmid curing in *M. avium* strain LR25 could be done using these treatments and possibly at higher growth concentrations to achieve higher inhibition. For reasons not known, colonies growing on pyruvate medium (MGAP agar) were not viable when transferred to MGT medium. These treatments induced pigmentation in *M. avium* strain LR25 at a frequency higher than that seen in the control. However, this phenomenon may not be correlated with plasmids.

Recently, DNA homology studies have indicated that *M. avium* strain LR25 and strain LR163 are not isogenic (McFadden, personal communication). Thus it is possible that the traits chosen for identifying putative cured derivatives of strain LR25 were not indicative of plasmid loss and that growth at 43°C in this strain may not be correlated to the presence of plasmids.

ELECTROPORATION

ELECTROPORATION OF *M. SMEGMATIS*

Mycobacterium smegmatis strain $mc^2 6$ was not transformed with pYUB12 by electroporation using the same parameters as those used by Snapper, et al., (1988). Increasing the number of pulses to two with a 10 sec delay, decreasing the volume of cells by 60%, and increasing the phenotypic lag period to 14 hours led to a very low transformation efficiency similar to that reported by Snapper, et al. (1988). The lack of death due to the elecrical pulses on the cells may be due to the complex cell wall structure of mycobacteria. Increase seen in the number of colony forming units/ml of cell suspension after the electrical pulses may be due to the disruption of cell clumps.

The kanamycin-sensitive cured derivative (strain VT307) showed a transformation frequency of $1 \times 10^5/\mu g$ pYUB12 and higher that that of strain $mc^2 6$. However, that value was within the range observed by Snapper et al. (1988) with an electroporation-proficient mutant. Evidently electroporation and selection of kanamycin-resistant selects such mutants. Expression of resistance to kanamycin occurred within 30 minutes after electroporation of *Mycobacterium smegmatis* strain $mc^2 6$ with pYUB12. Spontaneous mutants of *Mycobacterium smegmatis* strain VT307 occured at a frequency of less than 1×10^{-9}/cell. Natural competence in the presence of pYUB12 leading to kanamycin resistance was absent in strain VT307.

ELECTROPORATION OF *M. AVIUM* STRAIN LR163

Transformation of *M. avium* strain LR163 with pYUB12 was not successful under the conditions used here. There was no cell death due to the electrical pulses probably due to the complex cell wall of the mycobacteria. Strain LR163 did not show natural competence in the presence of pYUB12. The spontaneous occurence of kanamycin-resistant mutants of this strain was in a range of $1x10^{-9}$/cell. This frequency which was 100-times higher than that seen in strain VT307 makes strain LR163 unsuitable for electroporation with with pYUB12 when kanamycin is used as a marker. Future studies will therefore require a marker other than kanamycin resistance. Since strain LR25 and LR163 may not be isogenic, plasmids from LR25 cannot be used to transform LR163. The lack of transformation seen may be due to the complex cell wall and using methods to weaken the cell wall, or protoplast formation, may lead to sucesssful transformation. Another reason that transformants were not observed may be due to the restriction or modification of strain LR25 DNA in strain LR163 (Crawford, et al., 1985).

CURRICULUM VITAE

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EMPLOYMENT OBJECTIVES

Research scientist in microbiology

PERSONAL DATA

Born: November 18, 1965

EDUCATION

1990	Virginia Polytechnic Institute and State University Blacksburg, Virginia M.S. Microbiology, December 1990
1987	Virginia Polytechnic Institute and State University Blacksburg, Virginia B.S. Biology, August 1987

POSITIONS HELD

1987 - 1988	Graduate Teaching Assistant, General Biology, Department of Biology, VPI & SU, Blacksburg, VA.
1988 Summer	Graduate Research Assistant, Curriculum Transformation Project, College of Art's and Sciences; VPI & SU, Blacksburg, VA
1988 - 1989	Graduate Teaching Assistant, General Biology, Department of Biology, VPI & SU, Blacksburg, VA.
1989 Summer	Graduate Research Assistant, Curriculum Transformation Project, College of Art's and Sciences; VPI & SU, Blacksburg, VA.
1989 - 1990	Graduate Teaching Assistant, General Biology, Department of Biology, VPI & SU, Blacksburg, VA.
1989 -1990	Graduate Teaching Assistant, General Biology, Department of Biology, VPI & SU, Blacksburg, VA.
1989 - 1990	Graduate Student Representative, University Planning Co-ordinating Committee. Office of the Provost. VPI & SU.

M.S. RESEARCH

Mode of action of Clofazimine on *Mycobacterium avium* and *Mycobacterium intracellulare*
Evidence that the ability of MAIS to grow at 43°C is plasmid-encoded.

Major Advisor - Dr. Joseph O. Falkinham, III.
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DEPARTMENTAL SEMINARS

Identification And Cloning Of Virulence Genes in *Enterobacteriaceae* 1988.

Mode of action of the anti-leprosy drug Clofazimine

MEMBERSHIP IN PROFESSIONAL ORGANIZATIONS

American Society for Microbiology

LIST OF COURSES TAKEN IN GRADUATE SCHOOL

Nucleic Acids
Prokaryotic Regulatory Gene Mechanisms
Eukaryotic Gene Expression
Lambda Bacteriophage
Immunology I
Immunology II
Physiology of Microorganisms I
Physiology of Microorganisms II
Physiology of Microorganisms III
Biometry I
Biometry II
Graduate Microbiology Seminars

Present overall QCA = 3.3