PLASMID-INFLUENCED CHANGES IN *MYCOBACTERIUM AVIUM* CATALASE ACTIVITY

by

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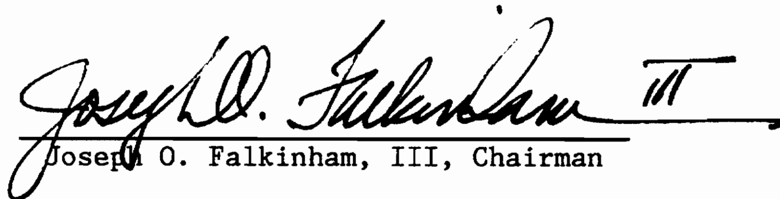

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(ABSTRACT)

A virulent Mycobacterium avium strain, LR25, which carries 3 plasmids (18, 28, and 165 kb) and grows at 43°C was compared to its plasmid-free, avirulent segregant, strain LR163, to investigate the basis for the latter's inability to grow at 43°C. The failure of mid-log phase cultures of strain LR163 to grow at 43°C was dependent upon the presence of high levels of culture aeration. In addition, highly aerated mid-log phase cultures of strain LR163 failed to grow at 37°C. By contrast, late-log phase cultures of strain LR163 were capable of growth when shifted to 43°C under highly aerobic conditions. Mid-log phase cells of strain LR163 had 30% of the catalase activity of mid-log phase cells of strain LR25 and were more susceptible to hydrogen peroxide (0.08% w/v). Catalase activities of late-log, early-stationary, and stationary phase cells of strain LR163 were significantly higher than mid-log phase cells. Catalase activity of strain LR25 was highest in cells of mid-log

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phase cultures, whereas the catalase activity of strain LR163 was highest in cells of stationary phase cultures. These data support the idea that plasmid-encoded genes influence *M. avium* catalase activity.

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INTRODUCTION

Mycobacteria of the *Mycobacterium avium* and *M. intracellulare* (*M. avium* complex) complex are opportunistic human and animal pathogens which cause pulmonary infections in humans similar to tuberculosis. Disseminated *M. avium* complex infection poses a particular threat to patients who are immunodeficient, such as those individuals suffering from Acquired Immune Deficiency Syndrome (AIDS).

Plasmid-encoded genes are implemented in the virulence of these organisms because of the observation that a high percentage of clinical isolates of *M. avium* complex strains contain plasmids. Also, the ability of *M. avium* complex organisms to grow at 43°C is correlated with the presence of plasmids. Growth at 43°C could serve as a marker for virulence in *M. avium* complex strains.

Studies of *M. avium* complex strains have shown that the organisms are highly resistant to oxidative killing by macrophages. During the oxidative burst of macrophages that occurs after phagocytosis of an organism, toxic forms of oxygen such as hydrogen peroxide, superoxide anions, and hydroxyl radicals are released. Possibly one factor involved in the virulence of these organisms is the production of a plasmid-

encoded catalase to break down hydrogen peroxide.

Because of the possible influence of plasmid-encoded genes on the virulence and ability of *M. avium* complex organisms to grow at 43°C, we sought to identify the basis for the ability of a plasmid-carrying virulent *M. avium* strain, LR25, to grow at 43°C and the failure of its plasmid-free avirulent derivative, strain LR163, to grow at 43°C.

REVIEW OF LITERATURE

Members of the genus *Mycobacterium* are pleomorphic rod-shaped, nonmotile, gram-positive, acid-fast organisms (Barksdale and Kim, 1977). The cell walls of these organisms have a high lipid content, which accounts for the acid-fast staining properties of the mycobacteria (Barksdale and Kim, 1977). The genus contains many representatives, rapid and slow growing, including many opportunistic and true pathogens (Wolinsky, 1979). The most distinctive pathogenic members of the genus are *M. tuberculosis* and *M. leprae*, which cause tuberculosis and leprosy, respectively.

Runyon (1980) developed a scheme for classification of mycobacteria associated with nontuberculous infections. He divided the organisms into four groups (I through IV). Members of Groups I, II, and III grow slowly, requiring seven or more days to produce a visible colony on solid medium. Group IV includes the rapidly growing representatives, which produce visible colonies on solid medium in less than seven days. Group I contains the photochromogenic mycobacteria, which produce pigmented colonies after exposure to light. Group II includes scotochromogenic organisms which produce pigmented colonies without exposure to light. Group III are the nonphotochromogenic organisms which do not

produce a pigment, even upon exposure to light.

In the early part of the twentieth century, M. tuberculosis was considered the main pathogenic member of the genus Mycobacterium (Wolinsky, 1979). However, within the last 30 years, the pathogenic significance of mycobacteria other than M. tuberculosis has been recognized. Mycobacteria of the Mycobacterium avium and M. intracellulare complex (M. avium complex), are an example of a group of opportunistic pathogens whose incidence of infection has remained steady, while the incidence of tuberculosis has fallen (Wolinsky, 1979).

M. avium and M. intracellulare are closely-related Group III mycobacteria. Biochemical and serological methods have failed to distinguish the two species, therefore they are grouped together as the M. avium complex (Wolinsky, 1979). However, DNA-DNA hybridization studies have shown that M. avium and M. intracellulare represent two separate species. In fact, some strains carrying M. intracellulare serotypes have proven to be strains of M. avium, based on the results of their hybridization reactions (Baess, 1979).

Organisms of the M. avium complex are commonly found in the environment. They are isolated from soil (Brooks et al, 1984), water (Falkinham et al, 1980), and aerosols formed from the surface of waters (Wendt et al, 1980). Their high frequency of recovery (Falkinham et

al, 1980) or high numbers (Brooks et al, 1984) in southeastern waters and soils coincides with their high frequency of recovery from samples sent to state tuberculosis diagnostic labs (Good, 1980; Good and Snider, 1982) and with the high frequency of persons reacting to antigens prepared from *M. avium* complex organisms (Edwards et al, 1969; Edwards, 1970). Members of this group of slow-growing opportunistic pathogens cause pulmonary infections (mycobacteriosis) in humans (Wolinsky, 1979), and pose a particular threat to individuals who are immunodeficient (Blaser and Cohn, 1986). Patients who suffer from Acquired Immune Deficiency Syndrome (AIDS) are at a risk for *M. avium* complex infection (Blaser and Cohn, 1986). Disseminated *M. avium* complex disease is the third most common opportunistic infection in patients with AIDS; only *Pneumocystis carinii* pneumonia and cytomegalovirus infection are more prevalent (duMoulin and Stottmeier, 1986).

Evidence that *M. avium* complex organisms recovered from the environment are a source of human infection emerged from the demonstration that *M. avium* complex aerosol isolates (and not those from soils or waters) share common characteristics with clinical isolates from patients with mycobacteriosis (Fry et al., 1986). Growth at 43°C, growth without albumin/oleic acid enrichment,

resistance to cadmium or streptomycin (Fry et al., 1986), and the presence of plasmid DNA (Meissner and Falkinham, 1986) are prevalent among clinical and aerosol isolates and rare among soil and water isolates. Meissner and Falkinham (1986) demonstrated that 56% of clinical isolates and 75% of aerosol isolates possess plasmids, whereas only five to six percent of soil and water isolates possess plasmids. In addition, the data of Fry et al. (1986) show that growth of M. avium complex organisms at 43°C correlates with the presence of plasmids. This evidence suggests that plasmids may encode for the ability of M. avium complex organisms to grow at 43°C.

Little is known about the significance and function of plasmids in M. avium complex organisms (Gangadharam et al., 1988). However, research has proven that some functions of these organisms are plasmid-encoded. These include restriction and modification of mycobacteriophages (Crawford et al., 1981), variation in colony morphology from transparent to opaque forms (Mizuguchi et al., 1981), and resistance to mercury (Meissner and Falkinham, 1984) or copper (Erardi et al., 1987). Evidence that plasmids play a role in the virulence of these organisms is indicated by the results of Meissner and Falkinham (1986). They found that a high percentage (56%) of clinical isolates of M. avium complex strains

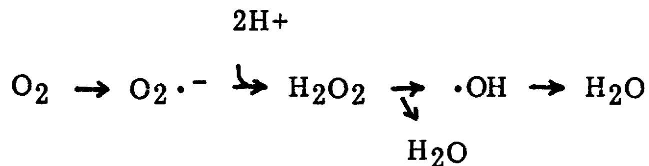
contain plasmids. In addition, Gangadharam et al.(1988) found that *M. avium* strain LR25, which has three plasmids of 18, 28, and 165 kilobases (kb) (Crawford et al., 1981), is of high virulence in beige mice, whereas its plasmid-free derivative, strain LR163, is of low virulence. Strain LR25 is able to grow at 43°C, whereas strain LR163 does not grow at this temperature (this study). These data suggest that the ability of *M. avium* complex organisms to grow at 43°C is correlated with virulence and that plasmids might play a role in the expression of both of these characteristics.

The possible influence of toxic forms of oxygen on *M. avium* complex infections was demonstrated by the observation that higher levels of both superoxide anion ($O_2 \cdot^-$) and hydrogen peroxide (H_2O_2) are released from mouse peritoneal macrophages infected with the plasmid-free derivative, strain LR163, compared to the plasmid-carrying parent, strain LR25 (Gangadharam et al., 1988). Perhaps the virulence of strain LR25 is related to its ability to escape the killing mechanisms of macrophages, which include the release of toxic forms of oxygen.

The reduction of oxygen which occurs during cellular metabolism and photooxidation in the environment (such as sunlight illumination of waters) (Cooper and Zika, 1983), results in the generation of toxic forms of

oxygen such as superoxide radicals ($O_2\cdot^-$), H_2O_2, and hydroxyl radicals ($\cdot OH$) (Hoffman et al., 1979). These derivatives are toxic to microorganisms (Imlay and Linn, 1988), including the mycobacteria (Sharp et al., 1985).

Consecutive univalent reduction of oxygen to its fully reduced form, water (H_2O), produces toxic forms of oxygen as shown below (Imlay and Linn, 1988):

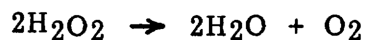


In addition, the toxic oxygen derivatives may be formed by other means (Gottschalk, 1986). For example, $O_2\cdot^-$ and H_2O_2 are formed from the autoxidation of reduced flavins, quinones, and iron/sulfur proteins, and H_2O_2 is formed from the dismutation of superoxide radicals.

Oxygen radicals and H_2O_2 are associated with chromosome deletions, single-stranded DNA breakage, and membrane damage (Imlay and Linn, 1988). The hydroxyl ion ($\cdot OH$) is the strongest oxidant of the oxygen derivatives and reacts with almost any organic molecule, including DNA (Imlay and Linn, 1988). It is theorized that the toxicity of other forms of oxygen, such as $O_2\cdot^-$ and H_2O_2, may result from the generation of hydroxyl radicals (Imlay and Linn, 1988). Hydroxyl radicals are formed in

the Fenton reaction, which involves the reduction of H_2O_2 by $O_2 \cdot^-$ in the presence of an iron complex, generating a hydroxyl radical (Imlay and Linn, 1988). Because more than one toxic form of oxygen may be present, it is difficult to determine which form is responsible for a given cytotoxic event.

Catalase, superoxide dismutase, and peroxidase are involved in the protection of microorganisms against toxic forms of oxygen (Fridovich, 1976). Superoxide dismutase is a metalloenzyme which catalyzes the dismutation of $O_2 \cdot^-$ to H_2O_2 and O_2. Peroxidase, together with a reduced electron carrier, breaks H_2O_2 down to H_2O. Catalase is an iron-porphyrin enzyme that catalyzes the decomposition of H_2O_2 to H_2O as shown below:



$$2H_2O_2 \rightarrow 2H_2O + O_2$$

The accumulation of this enzyme is associated with virulence in microorganisms. Virulent strains of *Staphylococcus aureus* (Mandell, 1975) and *Mycobacterium tuberculosis* (Jacket et al., 1978) have higher levels of catalase activity than less virulent strains.

It has been shown that H_2O_2 accumulates and stops the growth of microorganisms lacking catalase, such as *Mycoplasma pneumoniae* (Low et al., 1968). Yoshpe-purer and Henis (1976) demonstrated a correlation between the

amount of catalase in *E. coli* cells and their susceptibility to H_2O_2. Those strains with high catalase activity are less susceptible to H_2O_2 than those strains with low catalase activity. Catalase also protects DNA from strand scission (Brawn and Fridovich, 1981).

Catalase has been attributed to serving a role in the protection of microorganisms against oxidative killing by macrophages. During the macrophage's oxidative metabolic burst, $O_2\cdot^-$, H_2O_2, and $\cdot OH$ are generated (Haidaris and Bonventre, 1982). Virulent strains of *Nocardia asteroides* (Beaman et al., 1985) and *M. tuberculosis* (Beaman and Beaman, 1984) have high catalase activities (Beaman and Beaman, 1984; Beaman et al., 1985), and are able to survive and multiply within the host macrophages.

M. avium complex organisms produce catalase (Mayer and Falkinham, 1986a) and superoxide dismutase (Mayer and Falkinham, 1986b). Lygren et al. (1986) demonstrated that in *M. intracellulare*, there are catalases that also have peroxidase activity. Mycobacteria produce two classes of catalases, the T and M classes (Wayne and Diaz, 1982). The T class catalase is heat-labile and resistant to inhibition by 3-amino,1,2,4-triazole (AT), and the M class catalase is heat-stable and sensitive to inhibition by AT (Wayne and Diaz, 1982). The differences between the two classes in terms of thermal stability is

measured by heating cell-free extracts at 53°C for 50 minutes and measuring remaining catalase activity (Wayne and Diaz, 1982). Only T-catalases have been found in _M. avium_ and _M. intracellulare_ (Wayne and Diaz, 1982; Mayer and Falkinham, 1986[a]).

Attempts were made to determine if patterns of catalase activity resulting from gel electrophoresis could be used for classification of different mycobacteria. Nakayama (1967) attempted to use zymograms of catalase obtained from thin layer electrophoresis of a number of mycobacterial species as a means of differentiating them. However, he failed to detect enough differences between the catalases to make them useful in identification or classification of mycobacterial species. Valuable information concerning mycobacterial catalases has resulted from the visualization of catalase activity on polyacrylamide gels. Gruft and Gaafar (1971) discovered five different catalases within three species of mycobacteria studied. One catalase was inactivated by incubation at 68°C for 20 minutes. A second was partially inactivated by the above heat treatment, and the remaining three were resistant to the heat treatment. These results demonstrate that mycobacterial catalases are present in multiple molecular forms, such as the T and M catalases. Polyacrylamide gels have also been used for estimations of the molecular

weights of mycobacterial catalases (Gruft and Gaafar, 1974) and determination of susceptibility to heat inactivation and inhibitors such as AT (Mayer and Falkinham, 1986[a]). Polyacrylamide gel electrophoresis of cell-free extracts of strains of M. avium and M. intracellulare demonstrated two bands of activity, both of which are AT resistant and heat-sensitive (Mayer and Falkinham, 1986[a]).

M. intracellulare strains are highly resistant to oxidative killing by macrophages and H_2O_2 treatment (Gangadharam and Pratt, 1983; Gangadharam and Pratt, 1984). Gangadharam et al. (1988) demonstrated that the virulence of M. avium complex organisms is correlated with the presence of plasmids and their ability to trigger the release of oxygen metabolites from macrophages. Perhaps one factor involved in the virulence of these organisms is the production of a plasmid-encoded catalase.

Because of the possible influence of plasmid-encoded genes on virulence and the ability of M. avium complex organisms to grow at 43°C, we sought to identify the basis for the ability of a plasmid-carrying strain of M. avium, strain LR25, to grow at 43°C and the failure of its plasmid-free derivative, strain LR163, to grow at 43°C. Strain LR25 is virulent in beige mice (Gangadharam et al., 1988), grows at 43°C, and contains

three plasmids of 18, 28, and 165 kb (Crawford et al., 1981). Strain LR163 is avirulent in beige mice (Gangadharam, et al., 1988) and fails to grow at 43°C. Growth at 43°C could serve as a marker for virulence in these strains.

OBJECTIVE OF RESEARCH

The objective of the research was to identify the basis for the inability of strain LR163 to grow at 43°C.

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MATERIALS AND METHODS

Mycobacterial strains. *Mycobacterium avium* strain LR25 was isolated from a patient with mycobacteriosis at the Veterans Administration Medical Center in Little Rock, Arkansas (Crawford et al., 1981). Strain LR25 has three plasmids of 18, 28, and 165 kb (Crawford et al., 1981) and grows at 43°C. Its plasmid-free derivative, strain LR163, was isolated following neutral acriflavin exposure (Crawford et al., 1981) and fails to grow at 43°C. Both strains used in this study were obtained from the Veterans Administration Medical Center in Little Rock, Arkansas.

Media and preparation of inoculum. Mycobacterial cultures and inocula were grown in Middlebrook 7H9 medium (BBL Microbiology Systems, Cockeysville, MD) containing 1% (v/v) glycerol and 10% (v/v) oleic acid-albumin (OA) enrichment (MGE). The enrichment was prepared by mixing 8.5g NaCl and 50g bovine serum albumin, fraction V (Sigma Chemical Company, St. Louis, MO) in 1 L distilled water. After the NaCl and bovine serum albumin dissolved, 0.6 ml oleic acid (Sigma Chemical Company, St. Louis, MO) was added and the solution stirred for approximately 30 min. The solution was filtered sequentially through Metricel membrane filters (Gelman Sciences, Inc., Ann Arbor, MI)

with pore sizes of 5.0 µm and 0.45 µm, and was sterilized by filtration through a membrane with a pore size of 0.2 µm.

The inocula for the experiments were grown in MGE at 37°C with inversion one time daily in 18 x 150 mm screw-capped tubes to a turbidity of 25 (late-logarithmic phase) Klett Units (KU), measured with a Klett-Summerson colorimeter (Klett Mfg. Co., New York, NY) using a blue filter. The inocula were stored at 4°C for a maximum of two weeks.

Temperature shift growth experiments. Cells were inoculated (1% v/v) into 500-ml Nephelo Culture Flasks (Bellco Glass, Inc., NJ) containing 50 ml of MGE and were incubated in a Model G76 Gyrotory Water Bath Shaker (New Brunswick Scientific Co., Inc., New Brunswick, NJ) adjusted to 90 oscillations/min at 37°C. When the cultures attained mid-logarithmic (mid-log) growth, 25 ml of each culture was transferred aseptically by pipet to an identical sterile flask and incubated at 43°C in a second identical water bath shaker adjusted to 90 oscillations/min. The original flasks, each containing 25 ml of culture, were returned to the 37°C water bath.

Growth of cells was measured by increases in turbidity, total cell number, and viable cell number. Turbidity was measured with a Klett-Summerson colorimeter

using a blue filter. Total cell number was determined using a Petroff-Hausser Counting Chamber (Hausser Scientific, Blue Bell, PA). Viable cell counts, expressed as colony forming units per ml of culture (cfu/ml), were determined at 37°C on Middlebrook 7H10 agar medium (BBL Microbiology Systems, Cockeysville, MD) containing 1% (v/v) glycerol and 10% (v/v) OA enrichment, following dilution of the cultures.

Reconstruction experiments. After the initial shift experiments, four sets of reconstruction experiments were done. In the first set, cells of strain LR163 were inoculated (1% v/v) into a 500-ml Nephelo Culture Flask containing 50 ml of MGE and grown to mid-log phase (approximately 10 KU) at 37°C as described above. At this time, the entire culture was transferred aseptically by pipet to a second sterile Nephelo Culture Flask prewarmed to 37°C and then incubated as described above at 37°C.

In the second set, two Nephelo Culture Flasks, each containing 50 ml of MGE, were inoculated (1% v/v) with cells of strain LR163 and grown to mid-log phase at 37°C as previously described. One flask was transferred to an identical water bath at 43°C and incubated, and the other flask remained incubated at 37°C.

In the third set, a Nephelo Culture Flask

containing 50 ml of MGE was inoculated (1% v/v) with cells of strain LR163 and the culture was grown to mid-log phase as described above. Then, 25 ml of culture was removed using a sterile 25 ml pipet and transferred to a sterile Nephelo Culture Flask that had been prewarmed to 43°C. The volume was restored to 50 ml in both flasks by the addition of 25 ml of sterile MGE. The original flask remained at 37°C and the second was incubated in the 43°C water bath.

In the fourth set, two Nephelo Culture Flasks containing 50 ml of MGE were inoculated (1% v/v) with cells of strain LR25 and two Nephelo Culture Flasks containing 50 ml of MGE were inoculated (1% v/v) with cells of strain LR163. Immediately after inoculation, one flask of each strain was incubated in the 37°C water bath and the other was incubated in the 43°C water bath.

Increasing inoculum experiments. Experiments in which the inoculum size was varied were done after the reconstruction experiments. Cells of strain LR163 were inoculated into 500-ml Nephelo Culture Flasks containing 50 ml of MGE in the following percentages (v/v): 1%, 2%, 5%, 10%, and 20%. Growth of cultures was measured at 43°C as described above.

Measurement of catalase activity. Cells were inoculated

(1% v/v) into one liter screw-capped flasks containing 600 ml of MGE. The cultures were grown at 37°C to mid-log, late-log, early-stationary, or stationary phase. The cultures were shaken by hand one time daily to resuspend the cells. Cells were harvested by centrifugation at 10,000 x g for 30 minutes at 4°C, washed three times with 0.05 M sodium phosphate buffer (pH 7.0), and suspended in 20 ml of the above buffer. Cells were broken by two passages through a cold French Pressure Cell (Aminco J4-3398A American Instrument Co., Silver Sring, MD) at 18,000 to 20,000 lb/in^2. The lysates were centrifuged at 23,000 x g for 30 minutes at 4°C. The supernatant (crude extract) was stored at 4°C until used for the catalase assay. Assays were completed within 24 hours after preparation of crude extracts.

In addition to measurement of catalase activity for cells grown as described above, the influence of incubation temperature and culture aeration on the catalase activity of strain LR25 was also measured. To test the influence of growth temperature on the catalase activity of strain LR25, cells were inoculated 1% (v/v) into one-liter screw-capped flasks containing 100 ml of MGE. Cultures were grown to late-log phase at 37° and 43°C in a New Brunswick Model G76 water bath shaker oscillating at 90 oscillations/min. To test the influence of culture aeration on the catalase activity of

strain LR25, cultures were grown in a less aerated environment by inoculating cells (1% v/v) into 250-ml bottles containing 300 ml of MGE. A moderately aerated growth environment was obtained by inoculating cells (1% v/v) into one-liter screw-capped flasks containing 600 ml of MGE. Both types of cultures were grown to late-log phase at 37°C and shaken by hand one time daily to resuspend the cells. Cultures were grown in a highly aerated environment by inoculating cells (1% v/v) into one-liter screw-capped flasks containing 100 ml of MGE. The cultures were incubated in a New Brunswick Model G76 water bath shaker oscillating at 90 oscillations/min and grown to late-log phase at 37°C.

The method of Beers and Sizer (1952), which monitors the degradation of hydrogen peroxide at 240 nm, was used to assay the catalase activity of the crude extracts. Catalase activity is reported as units/mg protein ± standard deviation (one unit equals 1 µmole H_2O_2 decomposed per min). The catalase assay was done at 37°C and 43°C using a Model 102 Digital Spectrophotometer (Hitachi, Ltd., Tokyo, Japan). A temperature-controlled cell housing (Hitachi, Ltd., Tokyo, Japan) and a Model FJ constant temperature circulator (Haake, Inc., Saddle Brook, NJ) were used to maintain the temperature. Cell-free extracts were pre-warmed to the test temperature in a water bath before the assay was run.

The protein concentrations were determined by the method of Lowry et al. (1951) using bovine serum albumin fraction V (Sigma Chemical Company, St. Louis, MO) as the standard.

The data from the experiments studying the effect of culture age on the catalase activity of strains LR25 and LR163 were analyzed with Statistical Analysis System (SAS; SAS Institute, Inc., Cary, N.C.) using the general linear model below:

$$y_{ijkl} = m + a_i + b_j + s_k + as_{ik} + e_{ijkl}, \text{ where}$$

m = overall mean for catalase activity

a_i = fixed effect for strain

b_i = fixed effect for test temperature

s_k = fixed effect for length of culture incubation

as_{ik} = strain x length of culture incubation interaction

e_{ijkl} = random error associated with each $ijkl^{th}$ observation.

The data for the experiments studying the effect of incubation temperature on the catalase activity of strain LR25 were analyzed with SAS using the general linear model below:

$$y_{ijkl} = m + d_i + a_j + b_k + da_{ij} + db_{ik} + ab_{jk} + dab_{ijk} + e_{ijkl}, \text{ where}$$

m = overall mean for catalase activity

d_i = day effect

a_j = fixed effect for growth temperature

b_k = fixed effect for test temperature

da_{ij} = day x growth temperature interaction

db_{ik} = day x test temperature

ab_{jk} = growth temperature x test temperature interaction

dab_{ijk} = day x growth temperature x test temperature interaction

e_{ijkl} = random error associated with each ijklth observation.

The data were blocked by day because of daily variations in the catalase activities at each test temperature. The cause of the variation was not identified.

The data for the experiments studying the effect of culture aeration on the catalase activity of strain LR25 were analyzed with SAS using the general linear model below:

$$y_{ijk} = m + a_i + b_j + ab_{ij} + e_{ijk}, \text{ where}$$

m = overall mean for catalase activity

a_i = fixed effect for growth conditions

b_j = fixed effect for test temperature

ab_{ij} = growth condition x test temperature interaction

e_{ijk} = random error associated with each ijkth observation.

Isolation of the soluble fraction of crude extracts. For some experiments, catalase activity of the soluble cell fraction was measured. Following recovery of the crude extract (see "Measurement of catalase activity"), it was subjected to centrifugation at 100,000 x g for 60 min at 4°C to separate the cytoplasmic membrane (pellet) and soluble fractions.

Late-log phase temperature shift experiment. This experiment was performed as in the first temperature shift experiment with the exception that only cultures of strain LR163 were studied and cultures were grown to late log phase before the temperature shift.

Polyacrylamide gel electrophoresis. Soluble fractions were examined for bands of catalase activity in polyacrylamide tube gels using 0.022 M tris-0.18 M glycine electrode buffer (pH 8.2). A 7.5% (wt/vol) acrylamide resolving gel was prepared by mixing 3.75 ml of acrylamide:bisacrylamide (Bio-Rad, Richmond, CA) solution (30 g:0.8 g/100 ml distilled water, filtered, stored in a brown bottle at 4°C), 3.75 ml of 1.5 M Tris-Cl (pH 8.8), 7.5 ml of distilled water, 0.01 ml of TEMED (N,N,N',N'-tetra methylethylenediamine, Sigma, St. Louis, MO), and 0.05 ml of 10% (w/v) ammonium persulfate. The 4% stacking gel was prepared by mixing

0.65 ml of acrylamide:bisacrylamide (30 g:0.8 g), 1.25 ml of 0.5 M Tris-Cl (pH 6.8), 3.05 ml of distilled water, 0.005 ml of TEMED, and 0.025 ml of 10% (w/v) ammonium persulfate. Solutions were degassed for 10-15 minutes before addition ofTEMED and ammonium persulfate. Gels were poured at room temperature and were equilibrated in electrode buffer (pH 8.2) for one hour before protein samples were loaded. The crude extracts or soluble fractions were concentrated using a Speed Vac concentrator (Savant Instruments Inc., Hicksville, NY). Each tube gel was loaded with 30 ul (about 100 ug protein) of sample and 10 ul of sample buffer prepared by mixing 0.4 ml 1.5 M Tris-Cl (pH 6.8), 0.4 ml glycerol, 0.2 ml distilled water, and a few crystals of bromophenol blue.

Electrophoresis was carried out using a water-cooled tube gel apparatus (Model 3-1751A; Buchler Instruments, Fort Lee, NJ). Voltage was maintained at 150 V using a model EC103 constant-voltage power supply (EC Apparatus Corporation, St. Petersburg, FL). Electrophoresis continued until the bromophenol blue sample buffer ran out of the gel. Bands of catalase activity were localized in the gel by a negative staining method that was described by Gregory and Fridovich (1974) and later improved by Clare et al. (1984). The bands appear clear against a dark brown background. The

activity stain assay was done at 37°C and 43°C in water baths in 18 x 150 mm screw-capped tubes using reagents that were pre-warmed to the appropriate temperature.

Tube gels were scanned by a densitometer (Quick Scan, Jr.; Helena Labs Corp., Beaumont, TX) by setting the zero baseline on a clear catalase band and maximum absorbance on the brown stained gel background.

Hydrogen peroxide susceptibility. A modification of the method of Subbaiah et al. (1960) was used to measure the effect of hydrogen peroxide (H_2O_2) on mycobacterial colony-forming ability. Cells of strains LR25 and LR163 were inoculated (1% v/v) into 18 x 150 mm screw-capped tubes containing 10 ml of MGE and incubated at 37°C with daily inversion to suspend the cells. After attaining mid-log phase, 0.4 ml of each culture was removed and added to a 125-ml Erlenmeyer flask containing 7.6 ml of 0.05 M sodium phosphate buffer (pH 7.0) and 0.08% (w/v) H_2O_2. Controls lacking H_2O_2 were also prepared. The molar extinction coefficient (ε_{240}) value of 43.6 mol^{-1} cm^{-1} was used to prepare a standardized H_2O_2 stock solution, from which the 0.08% (w/v) H_2O_2 solution was prepared. Immediately and after 60, 120, and 180 min incubation in 37°C and 43°C water bath shakers adjusted to 90 oscillations/min, colony forming units/ml of serial dilutions in 0.05 M sodium phosphate buffer (pH 7.0) were

determined on M7H10 agar medium containing 1% (v/v) glycerol and 10% (v/v) OA enrichment. Colonies were counted after 15 days incubation at 37oC.

The data from the hydrogen peroxide susceptibility experiments were analyzed with SAS using the general linear model below:

$$yijklm = m + di + aj + bk + sl + abjk + asjl + dsil + eijklm,$$ where

m = overall mean for percent survival

di = day effect

aj = fixed effect for strain

bk = fixed effect for treatment

sl = fixed effect for temperature

abjk = strain x treatment interaction

asjl = strain x temperature interaction

dsil = day x temperature interaction

eijklm = random error associated with each ijklmth observation.

The data were blocked by day because of variations in the percent survivals at each test temperature which may have resulted from clumping of cells during plate counts.

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RESULTS

Effect of temperature shift on growth of strains LR25 and LR163. Preliminary experiments demonstrated that strain LR163, the plasmid-free derivative of M. avium strain LR25, failed to grow at 43°C on Middlebrook 7H10 medium containing 1% (v/v) glycerol and 10% (v/v) OA, though it grew at 37°C. Strain LR25 grew on that medium at both temperatures. To study the effect of incubation at 43°C on the growth of strains LR25 and LR163, a series of temperature shift growth experiments were done.

After a shift in incubation temperature from 37° to 43°C, mid-log phase cultures of strain LR25 continued to grow at about the same rate as cultures incubated at 37°C (Figure 1). Growth was monitored by increases in turbidity (Figure 1), total cell number, and viable cell number. In each experiment, changes in total cell number at 37°C and 43°C and viable cells paralleled the results of turbidity measurements (not shown). After a shift in incubation temperature from 37° to 43°C, the turbidity of mid-log phase cultures of strain LR163 fell dramatically, then remained steady (Figure 1). Surprisingly, mid-log phase cultures of strain LR163 failed to grow at 37°C after removal of one-half of the culture volume (Figure 1). Because the extent of aeration was increased following removal of half the culture volume, the failure

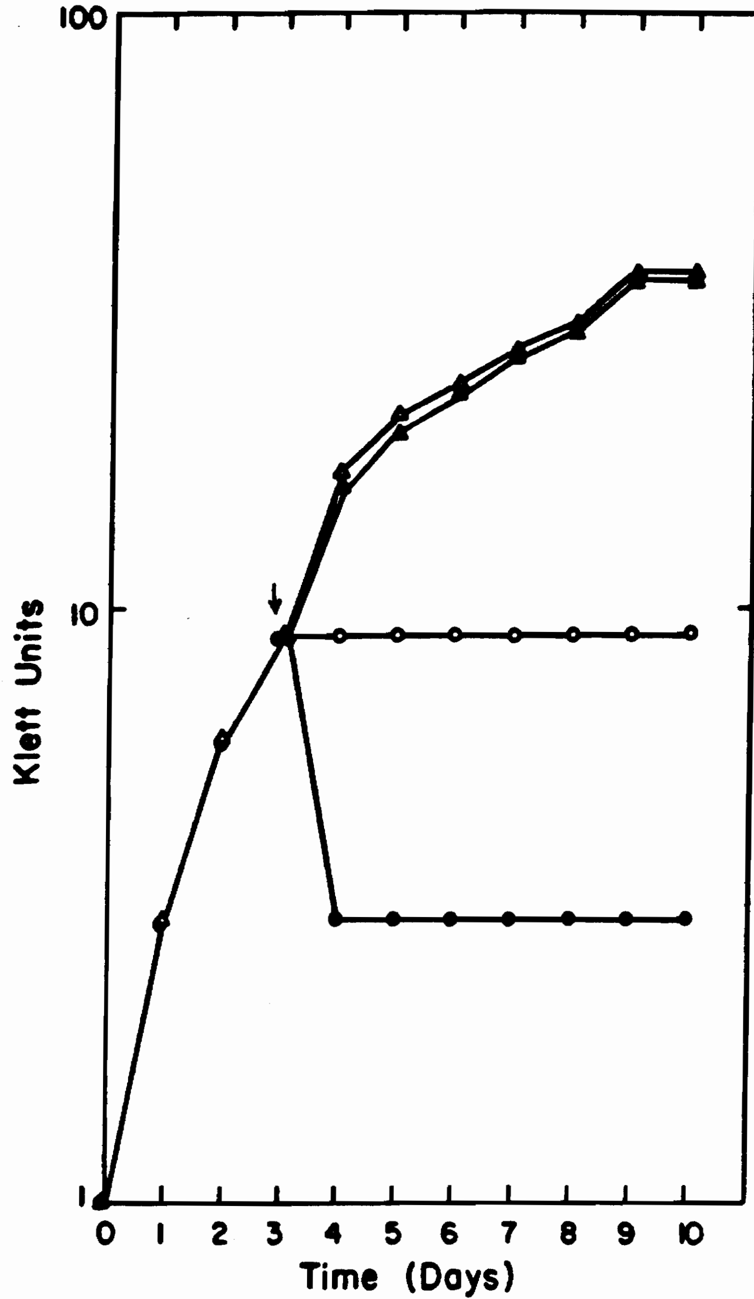

Figure 1. Growth of *M. avium* strains LR25 and LR163 in the temperature shift experiment. Cells of strains LR25 (△,▲) and LR163 (○,●) were incubated at 37°C and shifted to 43°C (▲,●) or remained at 37°C (△,○). The arrow indicates the point of the shift.

of strain LR163 to grow at 37°C suggested it might be oxygen-sensitive.

To explore the effect of reducing the culture volume and transfer by pipet on the growth of LR163, a number of temperature shift, culture transfer, and dilution experiments were performed. In the first set of experiments, the entire culture of strain LR163 (50 ml) grown to mid-log phase at 37°C was removed with a pipet, transferred to another flask and incubated at 37°C. After the transfer, mid-log phase cultures of strain LR163 continued to grow at 37°C (Figure 2). The growth of strain LR163 under these conditions indicated that sensitivity of the culture to transfer by pipet was not responsible for the failure of the strain to grow at 37°C (Figure 1).

In the second set of experiments, two individual 50-ml cultures of strain LR163 were grown to mid-log phase at 37°C. One of the cultures was transferred to a 43°C water bath and incubation of both cultures continued. Both cultures continued to grow as shown by increases in turbidity (Figure 3). In the third set of experiments, after transfer of 25 ml of a 50-ml culture of strain LR163 grown to mid-log phase at 37°C to another flask, the culture volume of both flasks was raised to 50 ml by the addition of 25 ml of sterile MGE. One flask was incubated at 37° and the other at 43°C. Cultures

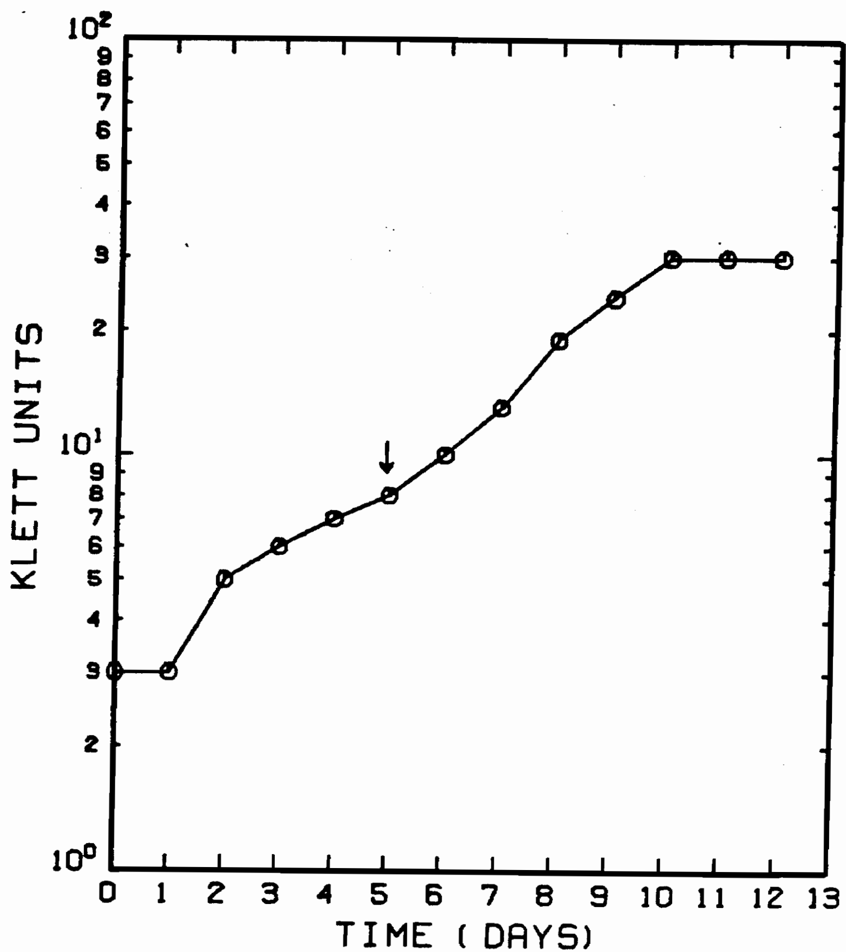

Figure 2. Test of sensitivity of M. avium strain LR163 to transfer by pipet. Cells (O) were incubated at 37°C before and after the point of culture transfer by pipette, which is indicated by the arrow.

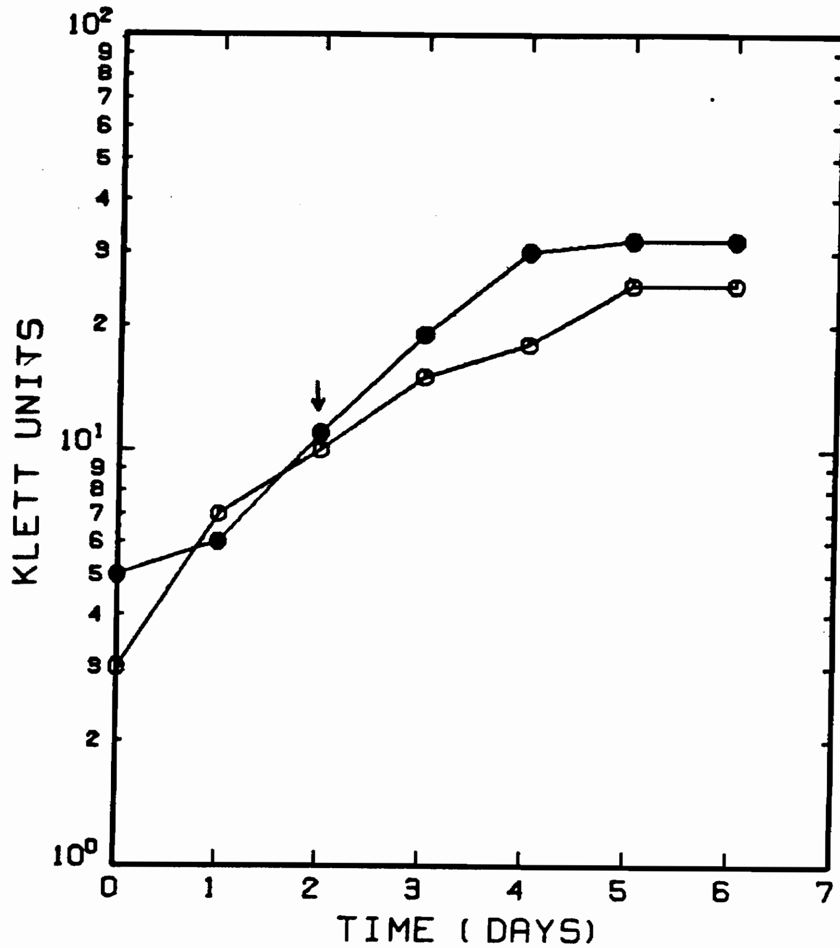

Figure 3. Effect of maintaining the culture volume on the growth of *M. avium* strain LR163 at 37°C and 43° C. Cells were incubated at 37°C and shifted to 43°C (○) or remained incubating at 37°C (●). The arrow indicates the point of the shift.

incubated at both temperatures continued to grow, as shown by increases in turbidity (Figure 4). The slight decrease in turbidity seen for both cultures at the time of the shift was due to the addition of 25 ml of MGE (Figure 4). The results of these latter two experiments support the hypothesis that removal of one-half of the culture volume, and hence increased aeration, was a factor in the failure of mid-log phase cultures of strain LR163 to grow at 37°C and 43°C.

In the last set of experiments, 50-ml cultures of both strains LR25 and LR163 were incubated at 37°C and 43°C immediately after inoculation. Cultures of strain LR25 incubated at 37°C and 43°C grew, as did cultures of strain LR163 incubated at 37°C (Figure 5). Cultures of strain LR163 incubated at 43°C did not grow (Figure 5). This result (Figure 5) contrasts with the observation that mid-log phase cultures of strain LR163 in which volume, and hence aeration, was maintained, did grow at 43°C (Figure 4). However, the cell density of these two cultures was different (i.e. the cell density of the shifted cultures was higher than those cultures just inoculated).

Effect of increased inoculum size on the growth of strain LR163 at 43°C. If aeration and oxygen toxicity were factors in preventing growth of strain LR163 at 43°C in

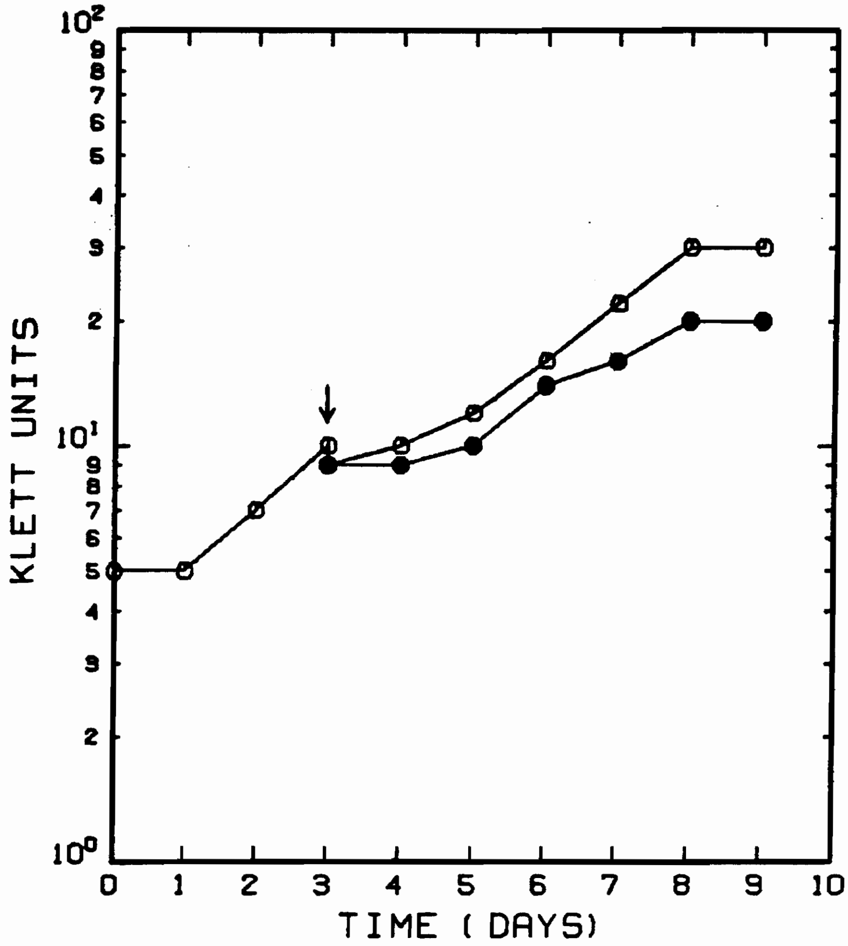

Figure 4. Effect of restoring the culture volume on the growth of M. avium strain LR163 at 37°C and 43°C following the temperature shift. Cells were incubated at 37°C and shifted to 43°C (●) or remained incubating at 37°C (○). The arrow indicates the point of the shift.

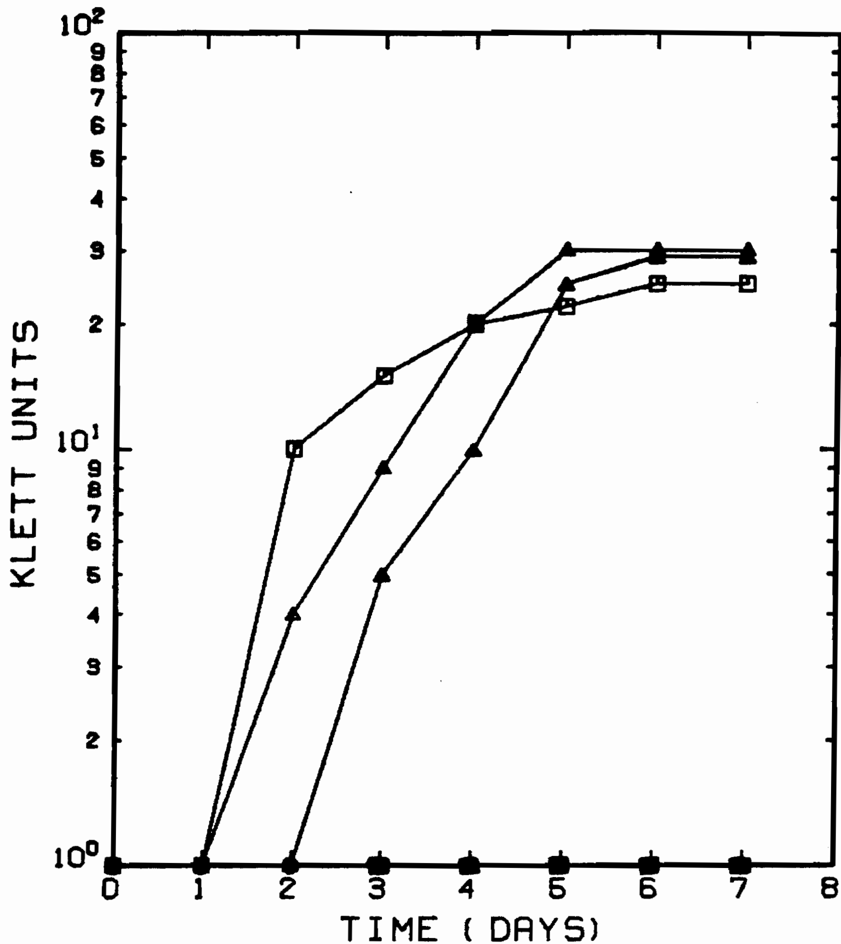

Figure 5. Effect of incubation at 43°C immediately following inoculation on growth of *M. avium* strains LR25 and LR163. Cells of strains LR25 (△,▲) and LR163 (□,■) were inoculated and incubated at 37°C (△,□) or 43°C (▲,■).

freshly inoculated cultures, addition of a larger inoculum might permit growth at 43°C. The rationale of this approach was that a high cell density can be protective against oxygen toxicity (Krieg and Hoffman, 1986). Cultures inoculated to a final percentage of 2% (twice standard inoculum), 5%, 10%, and 20% (v/v) grew slightly, as indicated by increases in turbidity (Figure 6), but quickly entered stationary phase. There was no correlation between higher inoculum volume and increased growth. Though growth of strain LR163 under these conditions was not equal to that of strain LR163 at 37°C (Figure 5), a larger inoculum did permit some growth, suggesting that oxygen toxicity may indeed play a role in the failure of strain LR163 to grow at 43°C and that the presence of a higher cell density may provide protection against toxic derivatives.

Catalase activity of strains LR25 and LR163. Because of the role of catalase in protection of microorganisms against toxic oxygen metabolites (Beaman and Beaman, 1984), the catalase activity of strains LR25 and LR163 was measured. Enzyme activity of cells of mid-log phase cultures was measured at 37°C and 43°C to determine the effect of temperature on catalase activity. The lack of a strain x test temperature interaction in the general linear model discussed in the "Materials and Methods"

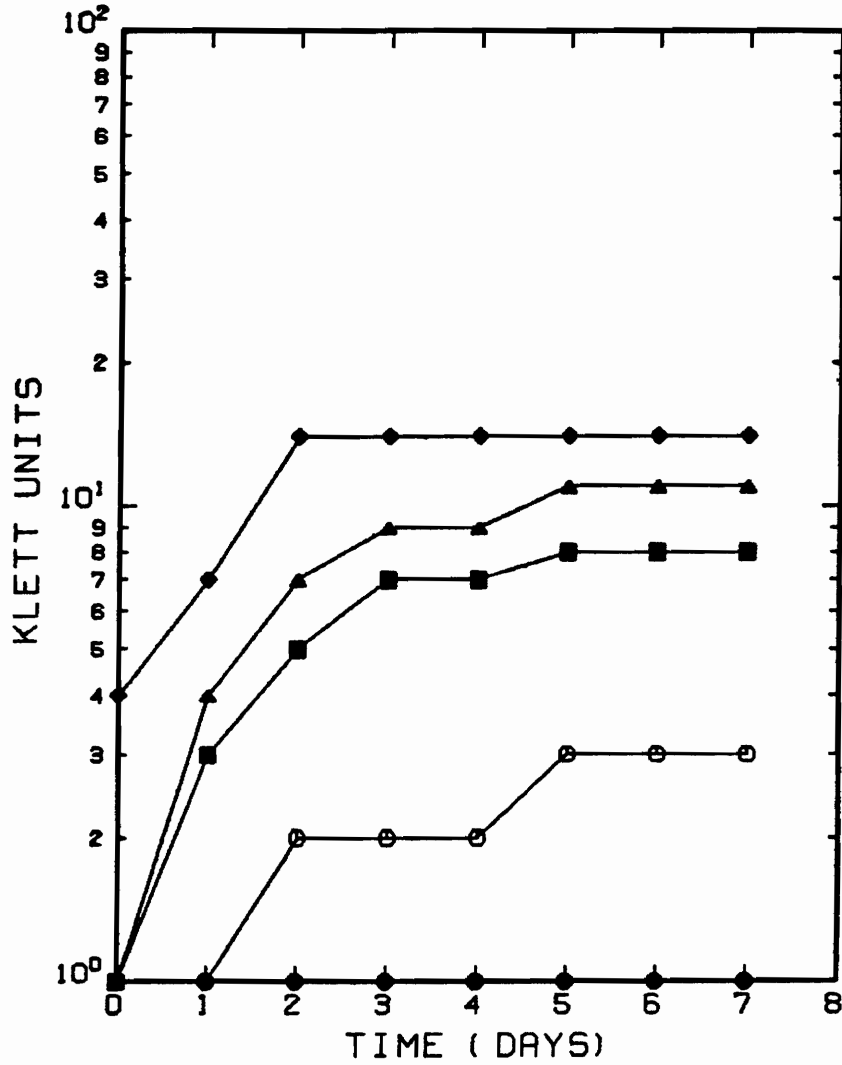

Figure 6. Effect of increased inoculum on the growth of *M. avium* strain LR163 at 43°C. Cultures were inoculated to a final percentage of 1% (standard inoculum) (●), 2% (twice standard inoculum) (○), 5% (■), 10%(▲), and 20% (◆) and were incubated at 43°C.

indicated that for all culture ages tested, the decrease in catalase activity measured at 43°C compared to that measured at 37°C for strain LR163 was no greater than the decrease in catalase activity of strain LR25 measured at 43°C compared to that measured at 37°C. Thus, some results are reported as significant across both test temperatures meaning that the difference between strains LR25 and LR163 in catalase activity measured at 37°C was proportional to the difference between the two strains in catalase activity measured at 43°C. Therefore the hypothesis that crude extracts of strain LR163, when assayed for catalase activity at 43°C, would lose a significantly greater percent of the catalase activity present at the 37°C assay temperature than would crude extracts of strain LR25 was rejected. The results in Table 1 show that crude extracts of mid-log phase cultures of strain LR25 had significantly higher mean catalase activities across the two test temperatures than strain LR163 (α=0.05; two-tailed T-test using the mean square error obtained from the model described in "Materials and Methods"). At each temperature, crude extracts of cells of strain LR163 had approximately 30% of the activity of extracts of cells of strain LR25. In addition, the mean catalase activity of both strains measured at 43°C was significantly lower than that at 37°C (Table 1; p=0.0001 and 0.0001, respectively).

Table 1. Catalase activity of crude extracts of cells of mid-log phase cultures cultures of *M. avium* strains LR25 and LR163.

Temperature of Assay	Catalase Activity[a] Strain LR25	Strain LR163
37°C	73.3 ± 17.3	21.9 ± 10.8
43°C	50.1 ± 11.5	11.9 ± 6.6

[a]Catalase activity in units/mg protein of mean ± standard deviation of 3 assays.

catalase activity of both strains was completely inhibited by 1 mM KCN and found to be in the soluble cell fraction in strains LR25 (Table 2) and LR163 (Table 3).

Effect of culture age on catalase activity of strains LR25 and LR163. Because growth stage influences catalase activity of *Nocardia asteroides* (Beaman et al., 1985) and the thermal stability of *M. avium*, *M. intracellulare*, and *M. scrofulaceum* catalase activity (Mayer and Falkinham, 1986), the effect of growth stage on catalase activity of cells of strains LR25 and LR163 was investigated as well. As culture age increased, the difference in catalase activity between extracts of strains LR25 and LR163 became smaller (Table 4). In mid-log phase cultures of strain LR25, the mean catalase activities measured at 37°C and 43°C were significantly greater than those of strain LR163 (Table 4; $\alpha = 0.05$; two-tailed T-test). However, late-log, early-stationary, and stationary phase cells of strain LR163 had approximately the same mean catalase activities across both test temperatures as those of cells of strain LR25. Note that the maximal catalase activity of strain LR25 was in mid-log phase cultures while that of strain LR163 was in stationary phase cultures.

Table 2. Localization of catalase activity of strain LR25.

Temperature of Assay	Catalase Activity[a] Crude Extract	Soluble	Pellet
37°C	47.6 ± 2.3	49.0 ± 11.8	<1.0
43°C	21.1 ± 8.9	26.9 ± 2.6	<1.0

[a]Catalase activity in units/mg protein of mean ± standard deviation of 2 assays.

Table 3. Localization of catalase activity of strain LR163.

Temperature of Assay	Catalase Activity[a] Crude Extract	Soluble	Pellet
37°C	22.6 ± 2.4	20.9 ± 4.8	<1.0
43°C	10.6 ± 2.3	5.4 ± 0.64	<1.0

[a]Catalase activity in units/mg protein of mean ± standard deviation of 2 assays.

Table 4. Effect of culture age on catalase activity of crude extracts of *M. avium* strains LR25 and LR163.

Culture Age	Temperature of Assay	Catalase Activity[a] Strain LR25	Strain LR163
Mid-log	37°C	73.3 ± 17.3	21.9 ± 10.8
	43°C	50.1 ± 11.5	11.9 ± 6.6
Late-log	37°C	48.3 ± 18.1	34.9 ± 13.4
	43°C	36.3 ± 12.5	22.4 ± 8.6
Early Stationary	37°C	68.4 ± 9.8	54.5 ± 12.9
	43°C	41.8 ± 16.0	23.8 ± 15.3
Stationary	37°C	53.7 ± 12.8	55.1 ± 14.2
	43°C	32.0 ± 2.8	45.5 ± 10.0

[a]Catalase activity in units/mg protein of mean ± standard deviation of 3 assays.

Effect of temperature shift of late-log phase cells on growth of strain LR163. Following a shift in incubation temperature from 37°C to 43°C, a late-log phase culture of strain LR163 continued to grow at both 37° and 43°C when culture volume was halved (Figure 7). This result was in contrast to the result of shifting mid-log phase cultures (Figure 1). Thus, it is possible that the significantly higher catalase activity of late-log phase cultures of strain LR163 compared to mid-log phase cultures (Table 4, p=0.008) permitted strain LR163 to grow at 37° and 43°C after the temperature shift in a highly aerated environment.

Effect of temperature of incubation on catalase activity of strain LR25. Because strain LR163, in contrast to strain LR25, failed to grow at 43°C (Figures 1 and 5), it was possible that a novel catalase activity was induced by incubation of cultures of strain LR25 at 43°C. However, the data in Table 5 show that growth of strain LR25 at 43°C failed to significantly induce additional catalase activity.

Effect of culture aeration on catalase activity of strain LR25. The extent of culture aeration influenced catalase activity of strain LR25. The mean catalase activity of the highly aerated culture measured at 37°C was

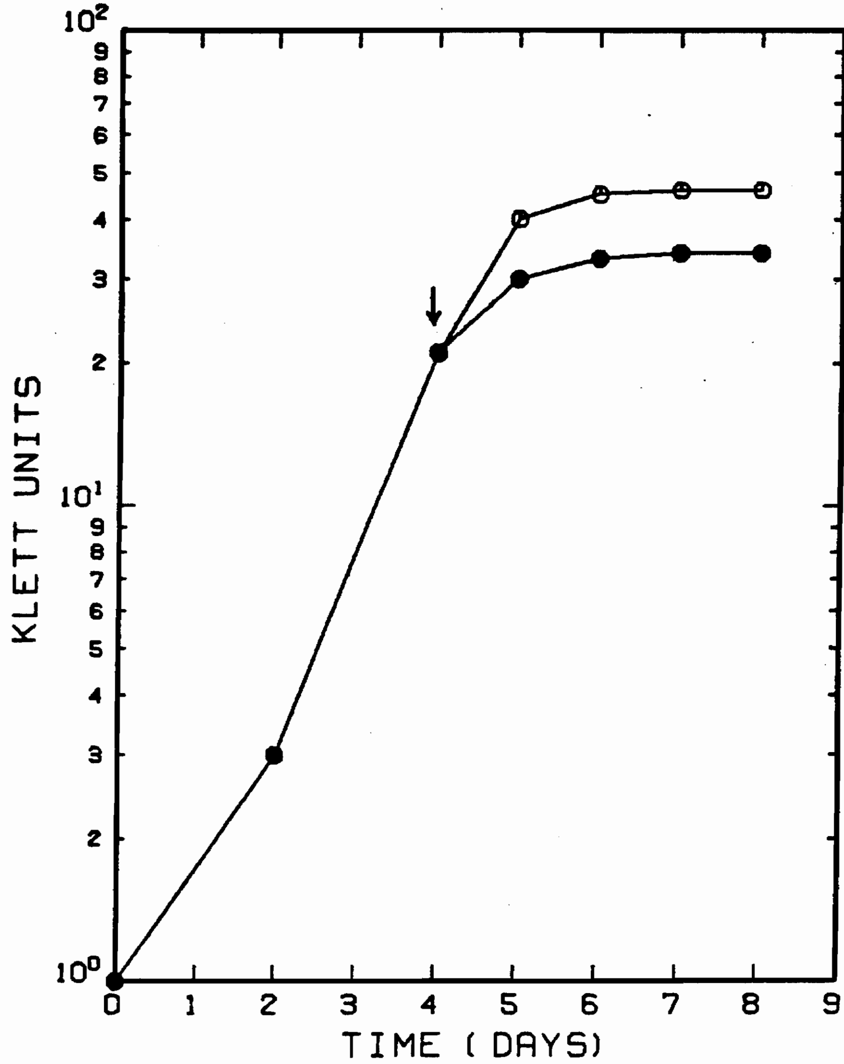

Figure 7. Effect of shifting late-log phase cells on the growth of M. avium strain LR163 at 37°C and at 43°C. Cells were incubated at 37°C and shifted to 43°C (●) or remained incubating at 37°C (○). The arrow indicates the point of the shift.

Table 5. Effect of growth temperature on catalase activity of crude extracts of *M. avium* strain LR25.

Temperature of Assay	Catalase Activity[a] Growth Temperature 37°C	43°C
37°C	64.2 ± 12.5	52.7 ± 13.3
43°C	35.2 ± 9.6	39.4 ± 13.4

[a]Catalase activity in units/mg protein of mean ± standard deviation of 2 assays.

significantly higher than those of the moderately aerated ($p=0.05$) and less aerated ($p=0.02$) cultures (Table 6). The mean catalase activity of the moderately aerated culture measured at 37°C was not significantly greater than that of the less aerated culture at 37°C ($p=0.4666$). The mean catalase activities at 43°C of cells grown under the less and moderately aerated conditions were not significantly different from the respective catalase activities at 37°C (Table 6; $p=0.6460$ and 0.2571, respectively). However, the catalase activity of the highly aerated culture measured at 43°C was significantly lower than that measured at 37°C ($p=0.005$) indicating a growth condition x test temperature interaction as seen in the general linear model described in the "Materials and Methods."

Electrophoretic separation of catalase activities of strains LR25 and LR163. The objective of electrophoretic separation of catalases of *M. avium* strains LR25 and LR163 was to determine if the higher catalase activity of strain LR25 was due to the appearance of a new catalase activity band in addition to the two catalase bands characteristic of *M. avium* strains (Mayer and Falkinham, 1986) and if the temperature of the staining procedure (37° or 43°C) affected the distribution of activity bands. Figures 8 and 9 show that the catalase activity

Table 6. Effect of culture aeration on catalase activity of crude extracts of *M. avium* strain LR25.

Temp of Assay	Catalase Activity[a]		
	Low Aeration[b]	Moderate Aeration[b]	High Aeration[b]
37°C	40.7 ± 7.6	48.3 ± 18.1	64.2 ± 12.5
43°C	36.9 ± 2.3	36.3 ± 12.5	35.2 ± 9.6

[a]Catalase activity in units/mg protein of mean ± standard deviation of 2 assays.

[b]See Materials and Methods.

Figure 8. Electrophoretic separation of catalase activities of *M. avium* strains LR25 and LR163 when stained at 37°C. Samples of crude extracts of mid-log phase cells, containing approximately 100 ug of protein, were stained for catalase activity at 37°C as described under "Materials and Methods." Lanes: (1) strain LR25; (2) strain LR163.

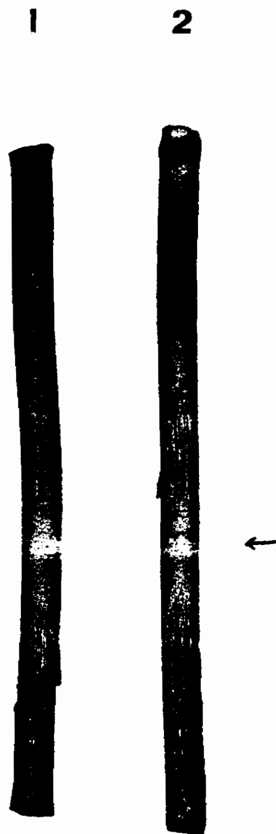

Figure 9. Electrophoretic separation of catalase activities of *M. avium* strains LR25 and LR163 when stained at 43°C. Samples of crude extracts of mid-log phase cells, containing approximately 100 ug of protein, were stained for catalase activity at 43°C as described under "Materials and Methods." Lanes: (1) strain LR25; (2) strain LR163.

of both strains LR25 and LR163 migrated as two bands. Though the two activity bands were not well separated (the densitometer detected two overlapping peaks for each strain at both test temperatures), there was no apparent difference in activity bands between the two strains at 37° (Figure 8) or at 43°C (Figure 9).

Hydrogen peroxide susceptibility of strains LR25 and LR163. If *M. avium* catalase activity was of biological significance, cells from mid-log phase cultures of strain LR25, which have more catalase activity than mid-log phase cultures of strain LR163, should be more resistant to hydrogen peroxide than cells from mid-log phase cultures of strain LR163. Mid-log phase cultures of strains LR25 and LR163 were both susceptible to 0.08% (w/v) H_2O_2 at 37°C and 43°C (Figures 10 and 11). However, the mean percent survival of cells of strain LR25 was significantly greater than the mean percent survival of cells of strain LR163 at both 37°C ($p=0.05$) and 43°C ($p=0.0043$).

Also, cells of strain LR25 were significantly more susceptible to 0.08% (w/v) H_2O_2 at 43°C than at 37°C ($p=0.001$). Surprisingly, though the survival of cells of strain LR163 was 100-fold less at 43°C compared to those exposed to 0.08% (w/v) H_2O_2 at 37°C, the differences were not significant ($p=0.235$). This may have been due to

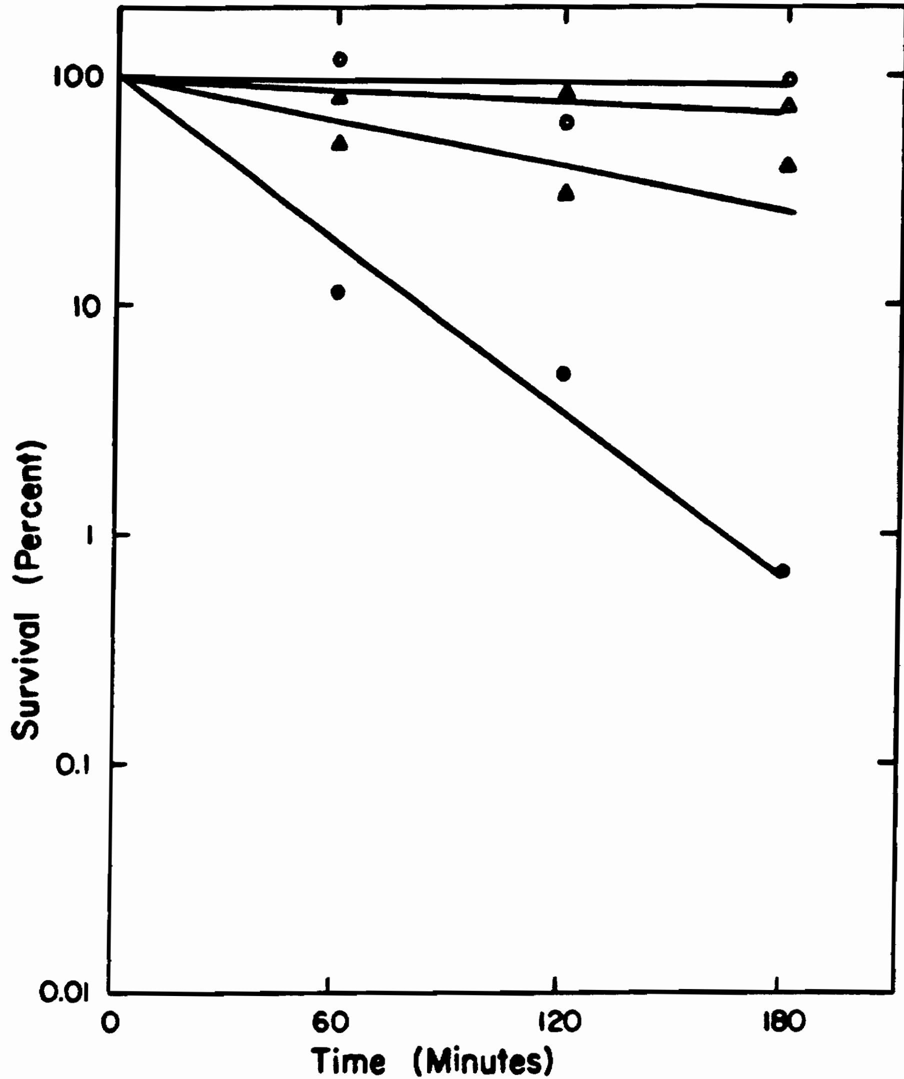

Figure 10. Susceptibility of M. *avium* strains LR25 and LR163 to 0.08% H_2O_2 at 37°C. Cells of strains LR25 (△,▲) and LR163 (○,●) were exposed to 0.05 M sodium phosphate buffer, pH 7.0 (△,○) and buffer containing 0.08% (w/v) H_2O_2 (▲,●) at 37°C.

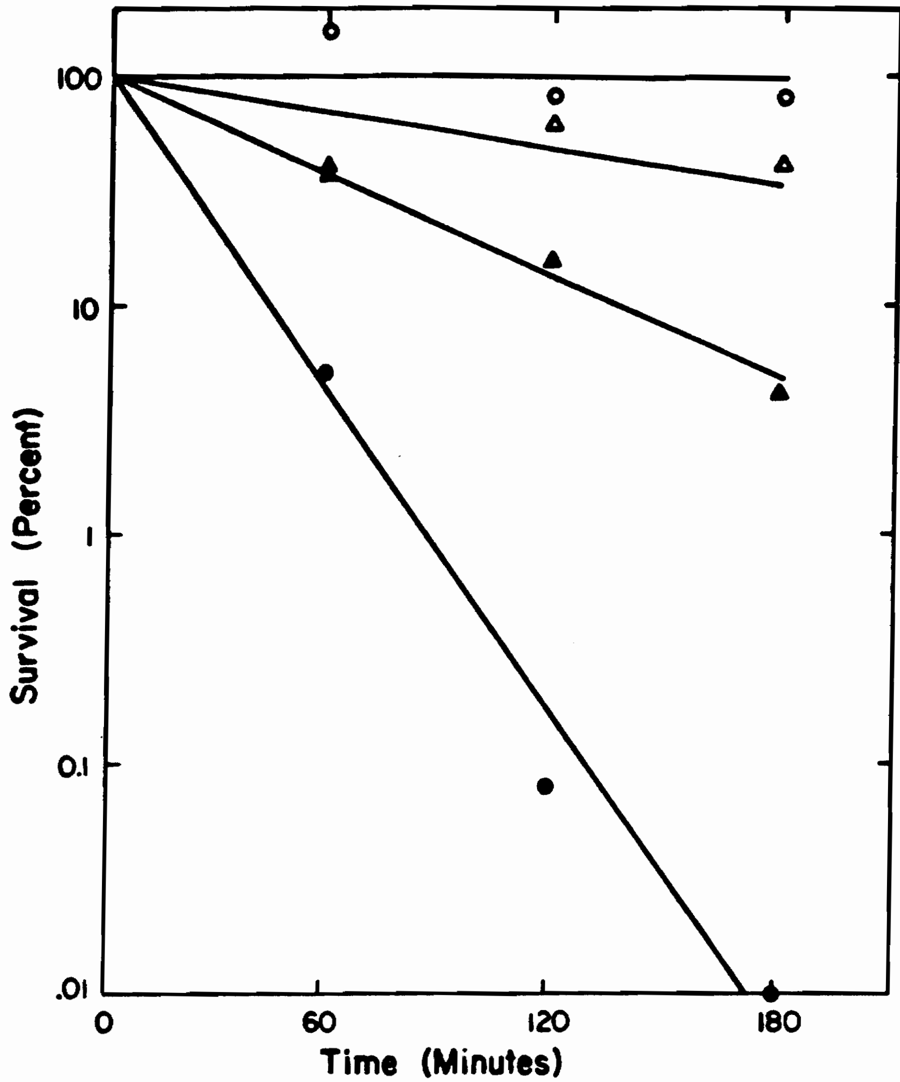

Figure 11. Susceptibility of M. avium strains LR25 and LR163 to 0.08% H_2O_2 at 43°C. Cells of strains LR25 (△,▲) and LR163 (○,●) were exposed to 0.05 M sodium phosphate buffer, pH 7.0 (△,○) and buffer containing 0.08% (w/v) H_2O_2 (▲,●) at 43°C.

daily variance in the data, resulting from clumping of cells during plate counts.

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DISCUSSION

The results of the growth studies suggested that the extent of culture aeration, here altered by an increase in the ratio of surface area to culture volume, may have been a factor in limiting the growth of strain LR163 at 37°C and 43°C. In the temperature shift experiments, mid-log phase cultures of strain LR163 failed to grow at both 37°C and 43°C after the culture volume was halved (Figure 1). By contrast, cultures of strain LR25 were capable of growth at 37°C and 43°C under all conditions. The reconstruction experiments demonstrated that mid-log phase cultures of strain LR163 grew at 37°C and at 43°C as long as the culture volume remained the same and aeration was not increased (Figures 3 and 4). Also, the failure of strain LR163 to grow at 43°C was not due to an experimental artifact, such as sensitivity to pipetting (Figure 2).

Catalase activity of both strains was influenced by culture age (Table 4) as observed for _Nocardia asteroides_ (Beaman et al., 1985). In _N. asteroides_, there is more catalase activity in early stationary phase than in log phase cells (Beaman et al., 1985). Mid-log phase cells of strain LR163 had significantly less catalase activity than did mid-log phase cells of strain LR25 (Table 4). The catalase activity of late-log, early-stationary, and

stationary phase cultures of the two strains were approximately equal (Table 4). The maximal catalase activity of strain LR25 appeared in mid-log phase cultures while that of strain LR163 appeared in stationary phase cultures (Table 4).

The low catalase activity of mid-log phase cultures of strain LR163 may have been one factor responsible for the strain's poor growth at 37° and 43°C in highly aerated cultures (Figure 1). This hypothesis is supported by the observation that strain LR163 grows well at 37° and 43°C when moderate aeration was maintained by not decreasing the culture volumes (Figures 3 and 4). In addition, late-log phase cultures of strain LR163, which had significantly more catalase activity than mid-log phase cultures (Table 4), were able to grow at 37° and 43°C in a highly aerated environment (Figure 7).

The results demonstrated that the catalase activities of strains LR25 and LR163 measured at 43°C were approximately 40% lower than those measured at 37°C (Table 2). The cultures of strain LR163 inoculated and incubated immediately at 43°C may have failed to grow (Figure 5) because the absolute level of catalase activity in the culture at 43°C was not high enough to allow growth in a moderately aerated environment. Cultures of strain LR163 which were grown to mid-log phase at 37°C before being transferred to 43°C and those

inoculated and incubated immediately at 37oC grew as the absolute value of catalase activity, even though it was most likely three times lower than that of strain LR25, was enough to allow growth in the aerated medium. Because increasing the inoculum volume, and hence the cell density and catalase activity of the culture, did not totally restore growth at 43oC of a freshly-inoculated culture of strain LR163 to that at 37oC (Figure 6), other factors in addition to oxygen toxicity may be involved in the inability of strain LR163 to grow at 43oC.

The catalase activity of strain LR25 was influenced by the extent of aeration during growth. Increased aeration, which results in an increase in toxic forms of oxygen in the medium, might be expected to induce higher levels of catalase or increase enzyme stability. Cultures grown at high levels of aeration had more enzyme activity than those grown with less aeration (Table 5). Whether this was due to true enzyme induction or rather to an increase in enzyme stability awaits further investigation. Incubation temperature was not shown to influence catalase activity of strain LR25 (Table 5).

The biological significance of the increased catalase activity of strain LR25 was demonstrated by the fact that this strain was more resistant to 0.08% (w/v) H_2O_2 than was strain LR163 (Figures 9 and 10) at both

37°C and 43°C. The increased susceptibility of both strains to 0.08% (w/v) H_2O_2 at 43°C might be explained by the lower catalase activities of these strains at that temperature (Tables 1 and 2). In contrast to our results, Gangadharam and Pratt (1984) did not find a correlation between catalase activity and H_2O_2-susceptibility. However, they did not compare isogenic strains. Thus, the responses of their strains could have been influenced by other factors (e.g. H_2O_2-permeation and pigmentation) as they suggested.

Finally, the data provide a possible explanation for the greater virulence of strain LR25 when compared to strain LR163 (Gangadharam et al., 1988). Hydrogen peroxide is a known agent of intracellular killing of bacteria (Beaman and Beaman, 1984) and microbial catalases have been shown to enhance phagocytic survival (Beaman and Beaman, 1984). The greater catalase activity of strain LR25 could result in greater survival in phagocytes, which is necessary for virulence, and a lower release of H_2O_2 by phagocytes as observed by Gangadharam et al. (1988). Because no new bands of catalase activity were observed in extracts of strain LR25, it is unlikely that a plasmid encodes for a novel catalase. The highest mean catalase activity of strain LR25 was found during mid-log phase and that of strain LR163 was found during stationary phase. Therefore it is possible that a

plasmid-encoded gene product alters the regulation of catalase production.

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