Investigation of Catalase and Superoxide Dismutase

from

Mycobacterium avium, M. intracellulare and M. scrofulaceum

by

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(ABSTRACT)

Catalase and superoxide dismutase, but not peroxidase activity was detected in cell-free extracts of Mycobacterium avium, M. intracellulare and M. scrofulaceum (MAIS). The M. scrofulaceum isolates had the highest catalase activity, while both M. avium and M. intracellulare had significantly lower activities. The percentage of catalase activity remaining, after exposing cell-free extracts from late log grown cells to 53°C for 50 minutes allowed differentiation among all three species. Polyacrylamide gel electrophoresis of crude extracts demonstrated two bands of catalase activity in both M. avium and M. intracellulare extracts and four bands of activity in M. scrofulaceum extracts. These bands differed in their susceptibility to heat inactivation and inhibition by 3-amino-1,2,4-triazole. M. scrofulaceum strains, but not M. avium and M. intracellulare, demonstrated extracellular catalase activity. The susceptibility to H_2O_2 of 6 M. avium strains, differing in catalase activity and cell permeability, were tested. At a concentration of 0.02% H_2O_2, all M. avium strains were resistant, while differences in susceptibility were seen at 0.08% H_2O_2. Strains of low extract catalase activity and high H_2O_2 permeability were most susceptible. The superoxide dismutase activities of the MAIS strains tested

were similar and no species-specific differences could be discerned. Electrophoresis of crude extracts demonstrated a single band of activity for each of the MAIS strains. Extracellular superoxide dismutase activity was detected in four of six MAIS strains. The metal type of MAIS superoxide dismutase was indirectly determined by inactivation with KCN, NaN_3 and H_2O_2.

ACKNOWLEDGEMENTS

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CHAPTER 1

INTRODUCTION

The genus _Mycobacterium_ is composed of a heterogenous group of rapid and slow growing, pathogenic and nonpathogenic species (35, 37). These bacteria are nonmotile, nonsporeforming, pleomorphic, gram-positive, acid-fast bacilli (3). Clearly the most renowned species of the genus are _M. tuberculosis_ and _M. leprae_. As the number of cases of _M. tuberculosis_ infection have declined, recognition of infection due to other species of pathogenic mycobacteria has increased. A 1980 study by the Centers for Disease Control (Atlanta) reported that _M. avium_, _M. intracellulare_ and _M. scrofulaceum_ (MAIS) accounted for 23% of pathogenic mycobacterial isolates (12). During a 3½-year period, infections with _M. avium-intracellulare_ represented 27% of all mycobacterial infections seen in the Milwaukee metropolitan area (28). More recently, it has been reported that a substantial proportion of individuals suffering from acquired immune deficiency syndrome (AIDS) have disseminated _M. avium-intracellulare_ infection (8, 38, 40). Disseminated _M. avium-intracellulare_ infection also has been reported in homosexuals and drug abusers probably suffering from AIDS (13). Infection with MAIS is particularly troublesome because they have a high degree of resistance to most antituberculous drugs (25, 27).

Unlike _M. tuberculosis_, MAIS organisms are commonly found in the environment. They have been isolated from soil (6), water (9) and air (36). Studies have indicated a predominance of MAIS in waters and soils of southeastern compared to northeastern United States (6, 9). The higher frequency of recovery of MAIS group strains in the southeastern United States waters was due to the increased recovery of pigmented, catalase-producing strains (14). This suggests that pigmentation and catalase activity may serve some adaptive function in the warm, sunny southeastern environment. Because

others have shown that sunlight illumination of natural waters results in the generation of superoxide radicals (O_2^-) and hydrogen peroxide (H_2O_2) (7) which are toxic to microorganisms (1, 17, 39) including mycobacteria (29, 30), superoxide dismutase, catalase and peroxidase activities may serve an adaptive, survival function. These enzymes breakdown O_2^- and H_2O_2 by the following reactions.

(a) $2O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2$

(b) $2H_2O_2 \longrightarrow 2H_2O + O_2$

(c) $H_2O_2 \underset{AH_2}{\longrightarrow} 2H_2O + A$

In reaction (a), superoxide dismutase catalyzes the dismutaion of O_2^- to H_2O_2 and O_2. In reaction (b), catalase breaks down H_2O_2 to H_2O and O_2 and in reaction (c) peroxidase, with the aid of a reduced electron donor (AH_2), breaks down H_2O_2 to H_2O.

In addition to serving a physiological role in the aquatic environment, these three enzymes provide the primary defense against oxygen toxicity (4, 18, 39). The univalent reduction of oxygen results in the production of toxic intermediates; O_2^- and H_2O_2 (4, 17). In the presence of iron complexes, O_2^- can reduce H_2O_2 to form the highly reactive hydroxyl radical (OH$^\cdot$) (16, 23), which may be even more toxic to the cell (17). Superoxide dismutase, catalase and peroxidase enzymes are therefore important to the survival of oxygen-metabolizing microorganisms.

The production of these enzymes by microorganisms is also associated with virulence and their ability to survive oxidative killing by phagocytes. Virulent strains of Mycobacterium tuberculosis (20) and Staphylococcus aureus (22) have higher catalase activities than less virulent strains. Also virulent strains of

Nocardia asteroides (5) have both high superoxide dismutase and catalase levels. During the oxidative metabolic burst of macrophage, O_2^-, H_2O_2 and $OH^\cdot$ are generated (4, 15, 21, 32), yet the intracellular pathogens M. tuberculosis (4) and N. asteroides (5) are capable of surviving and multiplying within the host macrophage. Their resistance has been attributed to high catalase activity and the production and secretion of superoxide dismutase, which becomes associated with the cell surface (4, 5). Less virulent strains of N. asteroides, which have low catalase activity and no surface-associated superoxide dismutase are very susceptible to phagocytosis by macrophage (5). They are however, protected from phagocytic attack by the addition of exogenous catalase or superoxide dismutase (5). The addition of both catalase and superoxide dismutase provides even more protection (5).

MAIS organisms have been shown to produce catalase (33, 34), but no information is currently available on superoxide dismutase activity. One source reported the absence of peroxidase activity (31). Previous studies of M. intracelulare strains have demonstrated high resistance to phagocytosis by macrophage and high resistance to in vitro H_2O_2 (10, 11).

By determining the superoxide dismutase, catalase and peroxidase activities (intracellular and extracellular) of MAIS organisms and correlating these activities with susceptibility to H_2O_2 and O_2^-, it may be possible to determine if superoxide dismutase, catalase and peroxidase provide an adaptive survival value within macrophage host cells and the aquatic environment where O_2^-, H_2O_2 and $OH^\cdot$ are generated.

Epidemiological studies of MAIS are currently hindered because there are no distinct phenotypic characteristics which are capable of distinguishing

between MAIS species. MAIS isolates are commonly classified based on their pigmentation and urease and catalase activities. M. avium and M. intracellulare strains are either pigmented or not, urease- and catalase-negative (24, 35), while M. scrofulaceum strains are usually pigmented, urease-positive and catalase-positive (35). Intermediates of these biovars have also been isolated (19, 26). Even a numerical taxonomic analysis of numerous phenetic traits of slow growing mycobacteria failed to distinguish between M. avium and M. intracellulare strains (24).

Serological classification is also unable to accurately distinguish between MAIS. DNA hybridization studies by Baess (2) have demonstrated that some strains belonging to M. intracellulare serotypes actually belong to the M. avium and M. scrofulaceum species based on their hybridization reactions. This misclassification may be the reason for the inability to biochemically differentiate between M. avium and M. intracellulare.

OBJECTIVES

At the onset of this research the following objectives were put forward:

1)   Measure and partially characterize the catalase, peroxidase and superoxide dismutase activities of representative strains of <u>Mycobacterium</u> <u>avium</u>, <u>M.</u> <u>intracellulare</u> and <u>M.</u> <u>scrofulaceum.</u>

2)   Determine whether correlations exist between enzyme activities and susceptibility to H_2O_2.

3)   Determine if any unique, species-specific characteristics exist, which can be used to classify environmental and clinical isolates.

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CHAPTER 2

Catalase Activity and Its Heat Inactivation for Differentiation of

Mycobacterium avium, M. intracellulare and

M. scrofulaceum

INTRODUCTION

It is well established that members of the Mycobacterium avium, M. intracellulare and M. scrofulaceum (MAIS) group are etiological agents of human and animal disease (6, 16, 20). More recently, it has been reported that a significant frequency of individuals suffering from Kaposi's sarcoma (4) and acquired immunodeficiency (AIDS, 21) have disseminated M. avium-intracellulare infection. Because of the wide spread geographical distribution of MAIS organisms in the environment (5), their epidemiology is becoming increasingly important. Epidemiological studies of MAIS are currently hindered because there are no distinct phenotypic characteristics for identifying and distinguishing MAIS isolates from one another (8, 13, 15). Proof that phenotypic characteristics fail as distinguishing features of MAIS organisms for speciation was demonstrated by the fact that a number of strains identified by either biochemical or serological methods as representatives of M. intracellulare actually belonged to either the M. avium or M. scrofulaceum species on the basis of DNA:DNA hybridization reactions (1). We therefore sought to study in detail the catalase activity (EC 1.11.1.6) of representatives of the three MAIS species defined by DNA hybridization, to discern if any differences existed which would distinguish each species.

Previous studies of mycobacterial catalase have shown the existence of two different types; the T and M classes (18, 19). The T class is heat-labile and resistant to inhibition by 3-amino,1,2,4-triazole (AT), while the M class catalase is heat-stable and AT-sensitive (19). The difference in thermal stability between the two classes can be determined by heating cell-free extracts at 53°C and measuring remaining activity (18). After measuring catalase heat inactivation of

representatives of the three MAIS species, it became apparent that there were significant differences. M. scrofulaceum catalase was very resistant, while M. avium catalase was intermediate in heat susceptibility and M. intracellulare catalase was very sensitive to heat inactivation. By determining the percentage of heat resistant catalase remaining, after heating cell-free extracts at 53°C for 50 minutes, it was possible to clearly differentiate the MAIS complex strains into their respective species. This could prove to be very useful in the identification of MAIS isolates.

MATERIALS AND METHODS

Bacterial Strains. Strains of mycobacteria used in this study (Table 1) were obtained from the National Jewish Hospital, Denver, Colorado and from the Statens Seruminstitut, Copenhagen, Denmark. Species assignments in Table 1 are based on DNA:DNA hybridization results (1, 9).

Growth of Cells and Preparation of Crude Extracts. Cell cultures and inocula were grown in Middlebrook 7H9 medium (BBL Microbiology Systems, Cockeysville, MD.) supplemented with 0.44% (v/v) glycerol, 0.2% (v/v) dextrose and 10% (v/v) oleic acid-albumin (OA) enrichment. The latter was prepared by mixing 8.5g NaCl and 50g bovine serum albumin, fraction V (Sigma, St. Louis, MO.) in 1000 ml of distilled water. While mixing, 0.6 ml of oleic acid (Sigma #O-0750) was added and the solution was stirred overnight at 4°C. The following day, the solution was filtered successively through 5 um, 1.2 um, 0.45 um, and 0.2 um Metricel membrane filters (Gelman Sciences, Inc., Ann Arbor, MI.) and finally filter-sterilized by passage through a sterile 0.2 um Metricel membrane filter.

The inocula were grown in 16x150 mm screw-capped tubes containing 10 ml of the above medium at 37°C for approximately 9 days and stored for use at 4°C for a maximum of 2 months. Cells were inoculated (1% v/v) into 1 liter screw-topped flasks containing 300 ml of the enriched Middlebrook 7H9 medium and incubated, with screw caps loose, for 12 days at 37°C. Flasks were shaken by hand daily to resuspend the cells and aerate the medium. 300 ml of late log-phase cells were harvested by centrifugation at 10,000 x g for 10 minutes, washed once in 100 ml of 0.05 M sodium phosphate buffer, pH 7.0, and

resuspended in a final volume of 10 ml of this buffer. Cells were broken by one passage through a cold French pressure cell (Aminco J4-3398A American Instrument Co., Silver Spring, MD.) at 18,000 to 20,000 psi. The crude extracts were clarified by centrifugation at 23,000 x g for 30 minutes and the supernatants retained for enzyme assays. Catalase activity was stable under refrigeration for at least 7 days, but was considerably reduced upon freezing extracts at 0°C.

Cultures for determination of extracellular catalase activity were grown in 1 liter screw-capped flasks as previously described with the exception that OA enrichment was omitted and the length of incubation was increased to 21 days. After centrifugation at 10,000 x g for 10 minutes, to remove cells, 300 ml of medium was concentrated to 10 ml by ultrafiltration through a PM-10 membrane (Amicon, Danvers MA.). The concentrate was then dialyzed for 24 hours at 4°C against 0.05 M sodium phosphate buffer, pH 7.0 and centrifuged at 23,000 x g for 10 minutes.

Assay of Catalase Activity. Catalase activity was determined by the method of Beers and Sizer (2), which monitors the degradation of hydrogen peroxide at 240 nm. Heat inactivation of catalase was done in a water bath set at 53°C. The crude, cell-free extracts were diluted into tubes containing preheated 0.05 M sodium phosphate buffer (pH 7.0) and incubated for the desired length of time. Following incubation, the tubes were cooled in ice and then allowed to warm to 25°C before residual catalase activity was determined. Protein was determined by the method of Lowry et al. (12) with bovine serum albumin (fraction V) as the standard.

Polyacrylamide Gel Electrophoresis (PAGE). Samples of crude, cell-free extracts were examined for bands of catalase activity in polyacrylamide slab gels using 0.022 M Tris-0.18 M glycine electrode buffer (pH 8.2). A 7.5% acrylamide resolving gel was prepared by mixing 5 ml of acrylamide:bisacrylamide (Bio-Rad Richmond, CA.) solution (30g:0.8g/100ml, filtered, stored in the cold in darkness), 5 ml of 1.5 M Tris-HCl, (pH 8.8), 0.01 ml of TEMED (N,N,N',N'-tetramethylethylenediamine, Sigma, St. Louis, MO.), 9.8 ml of distilled water, and 0.2 ml of 10% (w/v) ammonium persulfate. The 4% spacer gel was prepared by mixing 2 ml acrylamide:bisacrylamide (30:0.8), 1.5 ml of 1.5 M Tris-HCl (pH 7.0), 0.015 ml TEMED, 11.36 ml distilled water, and 0.15 ml of 10% (w/v) ammonium persulfate. Solutions for both resolving and spacer gels were degassed for 5-10 minutes before the addition of the 10% ammonium persulfate and 5-10 seconds afterwards. Gels were poured at room temperature, washed with buffer after polymerization and allowed to equilibrate for one hour in the presence of electrode buffer before protein samples were loaded. Each well contained 60 to 80 ul of crude extract (50-55 ug protein) and 20 ul of a 10% (v/v) glycerol, 0.05 M sodium phosphate (pH 7.0), solution with a few crystals of bromophenol blue added as the running dye marker.

Electrophoresis was carried out using a vertical tank apparatus (SE 600 series; Hoeffer Scientific Instruments, San Francisco, CA.) cooled with running water. Voltage was maintained at 150 V using a constant-voltage power supply (Model EC103; EC Apparatus Corporation, St. Petersburg, FL.) until the bromophenol blue tracking dye reached the bottom of the gel, approximately 4 hours.

Staining for Catalase Activity. Bands of catalase activity were localized on polyacrylamide gels by a method which depends upon the peroxidation of diaminobenzidine. This method was first described by Gregory and Fridovich (7) and later improved by Clare et al. (3). The bands of catalase activity appeared colorless against a uniformly brown background.

For visualization of heat-resistant catalase bands, the crude extracts were heated at 53°C for 50 minutes and then samples were loaded on gels.

Treatment of the extracts with 3-amino-1,2,4-triazole (AT) was carried out on the polyacrylamide gels after being subjected to electrophoresis. The gels were soaked in a solution containing 0.11 M AT made up in 0.05 M sodium phosphate (pH 7.0) for 1 hour at room temperature. The gels were then rinsed twice with distilled water and stained for catalase activity by the above procedure.

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.

RESULTS

MAIS Catalase Activity. Total catalase activity of cell-free extracts of the MAIS strains is shown in Table 2. M. intracellulare and M. avium had very similar activities with average values of 34.7 $\pm$ 11.7 units/mg (average $\pm$ standard deviation) and 35.5 $\pm$ 14.6 units/mg, respectively. The catalase activity of M. scrofulaceum strains was much higher, 134.8 $\pm$ 41.7 units/mg. For comparison, the results of a whole-cell semi-quantitative catalase assay have been listed in Table 1.

Heat Inactivation of Catalase Activity. M. intracellulare homology group strains TMC 1406 and Manten 157, M. avium homology group strains TMC 724 and TMC 706, and M. scrofulaceum homology group strains TMC 1312 and TMC 1306 were subject to heat inactivation by incubation of cell-free extracts at 53°C from zero to four hours. A plot of residual activity versus time of incubation, for one representative of each species, is displayed in Figure 1. These curves are characteristic of the data found for other representatives of each homology group. Strain TMC 1312 demonstrated the greatest resistance to heat inactivation, strain TMC 724 was intermediate in resistance, and strain Manten 157 was the most sensitive. Catalase activity of all these strains was completely inhibited by 1 mM KCN.

An incubation period of 50 minutes was then chosen to characterize the remaining strains, because it provided us with residual catalase values that clearly fell into three separate groups (Figure 1). The M. intracellulare homology group had only 14.1 $\pm$ 7.9% residual catalase activity (average $\pm$ standard deviation), M. avium had 53.3 $\pm$ 7.4% and the highly resistant M.

scrofulaceum homology group retained 82.8 ± 6.7% of its total catalase activity. The ranges did not overlap. These differences were significant at the 5% level using the one-way-analysis-of-variance method and Fisher's protected LSD (17).

Influence of Culture Age on Catalase Heat-resistance. Culture age or the stage of growth at which the cells are harvested had a pronounced effect on the heat resistance of M. avium and M. intracellulare catalase activity (Table 3). The percentage of heat resistant catalase activity increased as the cells went from early log to late log and finally to stationary phase.

Electrophoresis of MAIS Catalase. Electrophoresis of crude extracts demonstrated two bands of catalase activity for M. intracellulare strain Mark Robert and M. avium strain 4443-1237 and four bands of activity for M. scrofulaceum strain TMC 1306 (Figure 2, lanes 1,3,5). The activity of bands 1 and 2 of M. intracellulare strain Mark Robert were both very sensitive to 53°C exposure (Figure 2, lane 2). By contrast, the activity of band 1 of M. avium strain 4443-1237 became more intense after heat treatment, while band 2 decreased (Figure 2, lane 4). The activity of bands 1 and 2 of M. scrofulaceum strain TMC 1306 responded to heat treatment as did the corresponding bands of the M. intracellulare strains, while bands 3 and 4 showed little change (Figure 2, lane 6). These patterns of catalase activity were the same for other MAIS representatives.

The influence of heat treatment and AT inhibition, on the activity of bands of catalase were measured by scanning the gels with a densitometer. The total area under each band is listed in Table 4 using arbitrary units. The effect

of heat treatment on the area of each MAIS catalase band corresponded with the trend seen in Figure 2.

Treatment of the gels with AT, prior to staining, had little effect on bands 1 and 2 of all MAIS strains tested (55 - 99% of activity in control, Table 4). In contrast, band 3 of M. scrofulaceum strain TMC 1312 was completely inhibited and band 4 was moderately sensitive (46% of activity in control, Table 4).

Extracellular Catalase Activity. Concentrated culture medium of M. scrofulaceum strains TMC 1312, TMC 1306 and TMC 1323 had catalase activities of 1.8, 2.1 and 5.7 units/mg of protein, respectively. In contrast, catalase activity could not be detected in culture medium concentrates of M. avium strain 13528-1079 and M. intracellulare strain Manten 157. The extracellular catalase activities of the M. scrofulaceum strains were sensitive to 1 mM KCN.

Polyacrylamide gel electrophoresis of concentrated culture medium from M. scrofulaceum strain TMC 1323, demonstrated a single band of catalase activity (Figure 3, lane 2), which correlated in mobility with band 4 from crude extract of the same strain (Figure 3, lanes 1 and 3). Exposure of the concentrated culture medium for 50 minutes at 53°C did not diminish the catalase activity (Figure 3, lane 4).

Table 1. Strain list and MAIS semi-quantitative catalase activity.

Species and Strain[a]		Reference	Semi-Quantitative Catalase[b]
M. avium			
M. avium	S. 2 TMC 724 (ATCC 25291)[c]	1	4
M. avium	S. 1 TMC 706 (ATCC 15769)[d]	1	8
M. intracellulare	S. 4 13528-1079	1	50
M. avium	S. 2 TMC 701	9	4
M. avium	S. 3 TMC 721	9	6
M. avium	S. 3 Wild Strain E46941/76	1	4
M. intracellulare	S. 6 Susook	1	16
M. intracellulare	S. 8 J1868	1	16
M. intracellulare	S. 8 Wild Strain V3435/77	1	46
M. intracellulare	S. 5 4443-1237	1	40
M. intracellulare			
M. intracellulare	S. 7 Manten 157	1	24
M. intracellulare	S.12 TMC 1405 (P42)	1	26
M. intracellulare	S.14 TMC 1403	9	29
M. intracellulare	S.16 TMC 1406[e]	9	31
M. intracellulare	S.14 ATCC 25169	1	26
M. intracellulare	S.19 Darden	1	20
M. intracellulare	S.16 Mark Robert	1	9
M. intracellulare	S.18 2219, Altman	1	28
M. scrofulaceum			
M. scrofulaceum	S.41 TMC 1312 (P29)	1	>120
M. intracellulare	S. 9 TMC 1306 (P31)	1	>120
M. scrofulaceum	S.27 TMC 1302 (P6)	9	>120
M. scrofulaceum	TMC 1323[f]	9	>120
M. scrofulaceum	S. 9 ATCC 19073, SSC 218	1	96

[a] Based on DNA homology (1, 9), S. = serotype.

[b] Bubble height in mm. (11)

[c] Type strain of M. avium.

[d] Suggested IWGMT working type.

[e] Type strain of M. intracellulare.

[f] Type strain of M. scrofulaceum.

Table 2. Catalase activity of cell-free extracts of MAIS strains.

Species/Strain	Total Assays	Total[a]	Catalase Percent Post 50' @ 53° C[b]
M. intracellulare			
TMC 1403	3	25.3 ± 13.5	16.9 ± 4.9
TMC 1406	3	29.4 ± 0.4	16.0 ± 3.6
Manten 157	3	42.0 ± 4.2	11.8 ± 4.4
TMC 1405	2	14.6 ± 4.0	16.7 ± 8.2
ATCC 25169	3	32.8 ± 8.3	5.1 ± 2.7
Darden	3	38.8 ± 4.6	17.3 ± 8.1
Mark Robert	4	47.1 ± 10.4	6.3 ± 1.0
2219, Altman	3	36.6 ± 10.6	6.8 ± 16.8
Average ±S.D.[c]	24	34.7 ± 11.7	14.1 ± 7.9 (2.0->32.8) [d]
M. avium			
TMC 724	4	32.7 ± 1.3	46.7 ± 2.3
TMC 706	2	24.7 ± 1.5	49.0 ± 4.0
13528-1079	2	42.0 ± 5.5	49.2 ± 5.6
TMC 701	2	8.3 ± 0.2	49.2 ± 5.6
TMC 721	2	24.0 ± 14.9	54.0 ± 12.8
E46941/76	2	25.9 ± 1.4	55.4 ± 7.4
Susook	3	42.9 ± 4.4	57.8 ± 5.7
J1868	3	37.0 ± 6.8	50.1 ± 9.7
V3435/77	3	62.5 ± 13.4	63.9 ± 2.9
4443-1237	4	34.5 ± 3.2	56.2 ± 2.6
Average ± S.D.	27	35.5 ± 14.6	53.3 ± 7.4 (41.3->67.1)
M. scrofulaceum			
TMC 1312	3	147.8 ± 19.3	85.3 ± 0.7
TMC 1306	4	99.8 ± 11.0	74.8 ± 1.2
TMC 1302	2	81.9 ± 9.0	78.2 ± 0.2
TMC 1323	2	157.2 ± 19.4	92.0 ± 2.1
ATCC 19073	3	189.0 ± 8.6	87.8 ± 1.9
Average ± S.D.	14	134.8 ± 41.7	82.8 ± 6.7 (73.6->93.4)

[a] Units/mg. Protein ± standard deviation.

[b] % residual catalase activity ± standard deviation.

[c] Calculated from individual values for all strains and replicates.

[d] Range of values observed.

Table 3. Influence of culture age on total and heat-resistant catalase.

Species/Strain	Culture Age (Days)[a]	Catalase Total[b]	% Heat-Resistant
M. intracellulare			
2219, Altman	6	39.6	8.3
	12	45.7	25.8
	19	26.5	56.6
Darden	6	48.9	5.5
	12	39.9	14.6
	19	48.4	20.5
M. avium			
J1868	6	33.0	58.8
	12	44.5	48.8
	19	22.2	87.8
V3435/77	6	50.2	55.4
	12	77.6	63.3
	19	47.4	88.8
Susook	6	50.2	53.0
	12	47.8	59.6
	19	40.6	66.7
M. scrofulaceum			
TMC 1312	6	133.5	87.4
	12	166.0	85.8
	19	207.6	91.1

[a] Culture age related to growth stage by absorbance (Klett photometer): 6 days (Early Log); 12 days (Late Log); 19 days (Stationary).

[b] Units/mg. protein.

Table 4. Influence of heat and AT on catalase activities separable by PAGE.

| Species/Strain | Treatment | Catalase Band[a] | | | |
		1	2	3	4
M. intracellulare					
Mark Robert	None	42	110	-[b]	-
	50' @ 53°C[d]	<1(<2)	30(27)	-	-
ATCC 25169	None	46	138	-	-
	50' @ 53°C	7(15)	7(5)	-	-
Darden	None	32	81	-	-
	50' @ 53°C	<1(<3)	37(46)	-	-
	AT[e]	17(55)	69(84)	-	-
M. avium					
4443-1237	None	30	78	-	-
	50' @ 53°C	56(187)	61(78)	-	-
TMC 724	None	20	69	-	-
	50' @ 53°C	63(315)	55(80)	-	-
Susook	None	26	76	-	-
	50' @ 53°C	50(192)	54(71)	-	-
	AT	25(96)	75(99)	-	-
M. scrofulaceum					
TMC 1306	None	16	52	20	40
	50' @ 53°C	<1(<6)	<1(<2)	15(75)	40(100)
ATCC 19073	None	32	90	11	70
	50' @ 53°C	<1(<3)	<-----31----->[c]		65(93)
TMC 1312	None	11	68	7	61
	50' @ 53°C	<1(<9)	<-----11----->		53(87)
	AT	6(55)	50(74)	<1(<14)	28(46)

[a] Gels were scanned with a densitometer, and the peaks obtained were cut out and weighed to give areas of arbitrary units. Values in parenthesis represent percentage of untreated control.

[b] No band detected.

[c] Bands 2 and 3 appeared as one broad band.

[d] Extracts exposed to 53°C for 50 minutes immediately before electrophoresis.

[e] Gels exposed to 0.11 M AT.
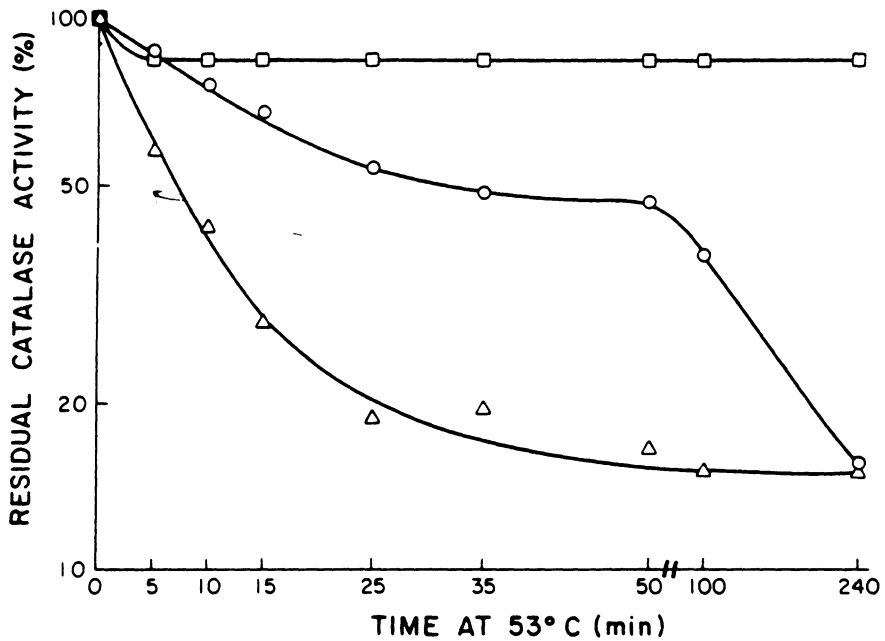
Figure 1.   Heat inactivation of MAIS catalase activity. M. scrofulaceum strain TMC 1312 (□), M. avium strain TMC 724 (0), and M. intracellulare strain Manten 157 (△). Crude extracts of late log phase cells were exposed to 53°C for 0 to 240 minutes and assayed for catalase activity.
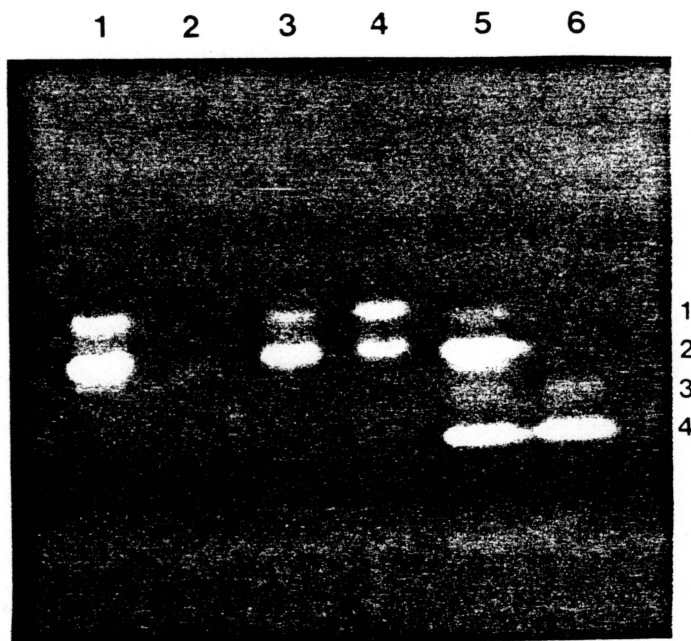
Figure 2.   Electrophoretic seperation of MAIS catalase activities. Samples of crude extracts of late log cultured cells, containing 50 to 55 ug of protein, were stained for catalase activity as described under "Materials and Methods". Lanes: (1) M. intracellulare strain Mark Robert, total catalase; (2) strain Mark Robert, after heat treatment (50' @ 53°C); (3) M. avium strain 4443-1237, total catalase; (4) strain 4443-1237, after heat treatment; (5) M. scrofulaceum strain TMC 1306, total catalase; (6) strain TMC 1306 after heat treatment.
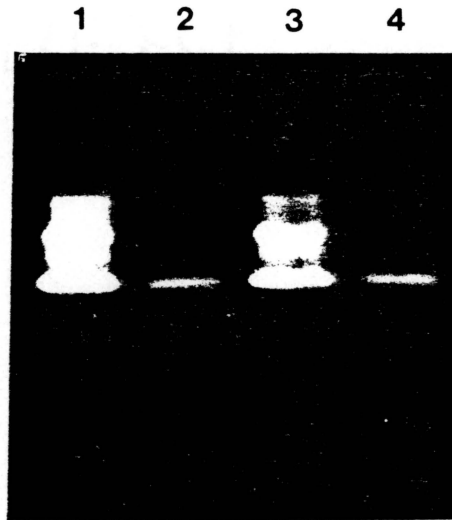
Figure 3.   Polyacrylamide gel electrophoresis of M. scrofulaceum strain 1323 demonstrating intracellular and extracellular catalase activities. Intracellular catalase activity obtained from cell-free extracts. Extracellular catalase activity obtained from culture medium concentrated 30-fold by PM-10 ultrafiltration. Lanes: (1) extract; (2) concentrated medium; (3) extract + concentrated medium; (4) concentrated medium after exposure for 50' @ 53°C.

DISCUSSION

Catalase activity has been an important biochemical test for characterizing mycobacteria for many years (11). The test often used is the semi-quantitative catalase test which measures bubble height in a culture tube after a solution of H_2O_2 is added. For MAIS organisms, a bubble height of usually greater than 45 mm is considered catalase-positive and anything less is catalase-negative (11). Using this whole cell, semi-quantitative test, M. scrofulaceum is usually catalase-positive, while both M. avium and M. intracellulare are catalase-negative (11). The results of this semi-quantitative catalase assay on the MAIS strains used in this study (Table 1) did demonstrate exceptions. Namely, both M. avium strains 13528-1079 and V3435/77 would be considered catalase-positive.

By using an assay (2) in which catalase activity in cell-free extracts is measured by the loss of H_2O_2, we were able to determine the specific activity (units/mg protein) of the representative MAIS strains (Table 2). The results of these assays demonstrate that semi-quantitative catalase activities do not predict activity in crude extracts in some strains. Though the M. scrofulaceum strains had very high catalase activities in crude extracts and M. intracellulare and M. avium strains had lower activities, only the M. scrofulaceum strains could be distinguished from the other species on the basis of total activity (T test, P = <0.01). There were no significant differences between M. avium and M. intracellulare activity values.

By heating cell-free extracts of MAIS strains at 53°C and measuring residual catalase activity after 50 minutes of incubation, significant differences among all three species were observed (Figure 1, Table 2). Wayne and Diaz (18)

may not have seen the difference in heat susceptibility of M. avium and M. intracellulare catalase activities due to the 4 hour incubation of extracts at 53°C. In our studies, at four hours of incubation, the percentage of heat-resistant catalase activities of M. avium amd M. intracellulare were indistiguishable (Figure 1).

The stage of growth at which the cells are harvested is an important variable influencing catalase heat stability (Table 3). In general, there was an increase in the percentage of heat-resistant catalase as the cells grew older. Cells harvested during stationary phase had much higher heat-resistant catalase activity. All of the strains reported in Table 2 were harvested after 12 days of growth, close to late log phase. The magnitude of the standard deviation in the average catalase values may have been due to variability in growth rate and hence cell age at harvest.

Visualization of catalase activity on polyacrylamide gels has generally led to poor resolution. Nakayama (14) determined zymogram patterns of catalase from a number of mycobacterial species in hope of differentiating them. His results however were inconclusive. In each of at least 8 species he detected 1 distinct band of activity, as well as additional, poorly defined traces of activity. Wayne and Diaz (18) detected 1 to 3 bands of activity in extracts from M. avium and M. intracellulare and 1 to 2 bands from M. scrofulaceum. Those studies utilized the H_2O_2 - KI system for visualizing catalase activity (14). When we applied the H_2O_2 - KI activity staining method used by Nakayama (14) and Wayne and Diaz (18) to the MAIS strain extracts, we could detect only 1 to 2 bands of catalase activity which rapidly dissappeared. We chose to abandon this method because it lacked stability and sensitivity. By utilizing a method which depended upon the peroxidation of diaminobenzidine for localizing bands

of catalase activity (3), we were able to consistently detect 2 bands of activity from representative strains of M. avium and M. intracellulare and 4 bands of activity from M. scrofulaceum (Figure 2). Bands 1 and 2 of all three MAIS species had identical mobility rates, while bands 3 and 4 of M. scrofulaceum migrated further.

The effect of heat treatment (50' at 53°C) on these bands of catalase activity can be seen in Figure 2 and Table 4. Activity bands of M. intracellulare strains were most sensitive to heat treatment with bands 1 and 2 both substantially reduced. This result was expected because extracts from M. intracellulare strains lost 85.9% of catalase activity when heated (measured by the Beers and Sizer method, 2, Table 2). Activity band 1 of all M. avium strains tested actually increased in intensity, as if being activated, while band 2 decreased. The heat activation seen in Band 1 could explain the intermediate resistance seen using the Beers and Sizer method (2). Bands 1 and 2 of M. scrofulaceum strains responded to heat treatment as did the corresponding bands of M. intracellulare; both being substantially reduced in intensity. Band 3 of M. scrofulaceum, which is only a minor band of catalase activity, displayed moderate resistance to heat inactivation. M. scrofulaceum catalase band 4, which was very heat resistant, probably is responsible for the greater heat resistance of M. scrofulaceum over strains of M. avium and M. intracellulare.

Because the two methods used for determining catalase activity (2, 3) have different sensitivities it was not possible to correlate activity bands (measured by densitometry) and activity in crude extracts (measured spectrophoto-metrically). Thus, the increase in heat-stability of M. avium and M. intracellulare catalase activities in extracts due to culture age could not be correlated with an increase in a specific band. However, the increased heat

stability of those species was not due to the production of the M. scrofulaceum heat-resistant enzyme (bands 3 and 4, Figure 2).

In a further attempt to characterize the activity bands separated by electrophoresis, AT-resistance was measured. Bands 1 and 2 of all MAIS strains tested were resistant to AT inhibition. Band 3 in the M. scrofulaceum strain was AT-sensitive and Band 4 in the same strain was moderately sensitive. According to Wayne and Diaz (19), M. intracellulare and M. avium strains produce only T catalase (heat-labile, AT-resistant), while M. scrofulaceum strains produce both the T and M catalase (heat-stable, AT-sensitive). Our results agree with this observation. Activity bands 1 and 2 in M. intracellulare and M. scrofulaceum and band 2 in M. avium are characteristic of T catalase, while activity bands 3 and 4 in the M. scrofulaceum strain tested are characteristic of M catalase. More work needs to be done to explain the apparent heat activation that occurs in activity band 1 of the M. avium strains.

In addition to high crude extract catalase activity, M. scrofulaceum, but not M. avium and M. intracellulare, demonstrated extracellular catalase activity. This catalase activity was inhibited by 1 mM KCN, indicating a traditional catalase enzyme. Electrophoresis of concentrated culture medium of M. scrofulaceum strain TMC 1323, demonstrated a single catalase band (Figure 3, lane 2), which was identical in mobility to band 4 of crude extract from the same strain (Figure 3, lanes 1 and 3). The fact that only band 4 was present in the concentrated culture medium, supports the conclusion that it is a true extracellular enzyme and not simply the result of cell lysis. The heat-resistance of this extracellular catalase (Figure 3, lane 4) is comparable with the high heat-resistance demonstrated by band 4 from M. scrofulaceum crude extract (Figure 2, lane 6).

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CHAPTER 3

Catalase and Permeation as Factors Affecting the

Susceptibility of Mycobacterium avium

to Hydrogen Peroxide.

INTRODUCTION

Mycobacterium avium is a member of a group of environmental pathogens (29) commonly isolated from soil (5), water (9) and air (28). Studies have shown that numbers of M. avium and related mycobacteria (M. intracellulare and M. scrofulaceum) are higher in waters (9) and soils (5) of the southeastern compared to the northeastern United States (5, 9). The higher frequency of recovery of MAIS group strains in the southeastern United States waters was due to the increased recovery of pigmented, catalase-producing strains (13). This suggests that pigmentation and catalase activity may serve some adaptive function in the warm, sunny southeastern environment. Because others have shown that sunlight illumination of natural waters results in the generation of hydrogen peroxide (H_2O_2) (6) which is toxic to microorganisms (1, 30) including mycobacteria (24, 25), catalase activity might serve an adaptive, survival function.

In addition to serving a physiological role in the aquatic environment, catalase may also provide protection against H_2O_2 generated in macrophages and neutrophils following phagocyctosis of mycobacteria (17, 26). In fact, addition of exogenous catalase has been shown to permit survival of some organisms in polymorphonuclear neutrophils in vitro (3, 20, 27). This observation suggests that high catalase activity, often associated with virulent strains of Mycobacterium tuberculosis (16), Nocardia asteroides (3) and Staphylococcus aureus, (20) is a virulence factor.

Studies of the in vitro susceptibility of microorganisms to H_2O_2 have demonstrated that catalase-negative strains or strains of lower catalase activity are usually more susceptible to the bactericidal effect of H_2O_2 than strains of

higher catalase activity (1, 12, 24). However, within the genus Mycobacterium, exceptions to this trend have been reported. M. tuberculosis strains recovered from South Indian and British tuberculosis patients had similar catalase activity, yet differed in their in vitro susceptibility to H_2O_2 (25). Overall M. tuberculosis is much more peroxide susceptible than M. intracellulare regardless of catalase activity (10). Strains of M. intracellulare which displayed catalase activity equal to or less than that of M. tuberculosis strains were much more resistant to 0.02% H_2O_2(10). To explain this reduced susceptibility, differences in the permeability to H_2O_2 of the cell wall of these organisms has been suggested (10, 25). Less permeability to H_2O_2 would correlate with greater resistance. A permeability barrier to H_2O_2 has also been suggested to explain differences in peroxide susceptibility of Bacillus species (8). The existence of a permeability barrier has been suggested as the reason for the naturally occurring antibiotic resistance of M. avium and M. intracellulare (7, 22, 23).

The subject of this report is the effect of H_2O_2 on strains of M. avium differing in catalase activity and cell permeability. An estimate of H_2O_2 permeability can be obtained by measuring semi-quantitative catalase activity of whole cells (19). This measures catalase activity of whole cells by the addition of exogenous H_2O_2 to a culture and measuring the column of bubbles produced. Strains of similar specific catalase activity and different semi-quantitative measurements should therefore differ in permeability.

MATERIALS AND METHODS

Bacterial Strains. Strains of Mycobacterium avium used in this study (Table 1) were obtained from the National Jewish Hospital, Denver, Colorado and from the Statens Seruminstitut, Copenhagen, Denmark. Species assignment was based on DNA:DNA hybridization results (2, 15).

Growth of Cells for Inocula. Stock cultures were maintainted on Löwenstein Jensen medium (BBL Microbiology Systems, Cockeysville, MD.) stored at 4°C. Inocula of M. avium used in this study were grown by aseptically transferring a loop of cells to 16 x 150 mm screw-capped tubes containing 10 ml of Middlebrook 7H9 medium (BBL Microbiology Systems) supplemented with 0.44% (v/v) glycerol, 0.2% (v/v) dextrose and 10% oleic-albumin (OA) enrichment. The latter was prepared as previously described (Chapter 2). The inocula were grown at 37°C, vortexed daily for approximately 9 days and stored for use at 4°C for a maximum of 2 months.

Semi-Quantitative Catalase Test. This crude quantitation of mycobacterial catalase activity was performed essentially as described by Kubica (19). For each M. avium strain to be tested, 3 ml of Dubos broth (BBL Microbiology Systems) supplemented with 0.44% (v/v) glycerol, 0.2% (v/v) dextrose and 10% (v/v) OA enrichment was inoculated (two drops) and incubated at 37°C for 9 days undisturbed. From these 9-day cultures, 0.1 ml of sedimented cells were transferred to the surface of 5 ml Löwenstein-Jensen butts (18 x 150 mm screw-capped tube). The inoculated butts were incubated at 37°C, with screw caps loose, for 2 weeks. Catalase activity was measured by adding 1 ml of a 1:1

mix of 30% H_2O_2 (Sigma, St. Louis, MO.) and 10% (v/v) Tween 80 to the surface of the culture growth. After 5 minutes the height of the column of foam above the surface of the medium was measured in millimeters.

Hydrogen Peroxide Susceptibility Test. The effect of H_2O_2 on mycobacterial viability was tested by a modification of the bactericidal test described by Subbaiah et al. (25). Each strain to be examined was inoculated (1% v/v) into 10 ml of Middlebrook 7H9 medium supplemented with 0.44% (v/v) glycerol, 0.2% (v/v) dextrose and 10% (v/v) OA enrichment. The cultures were grown at 37°C for 9 days with daily vortexing. 0.2 ml of culture were then removed and incubated at 37°C with 3.8 ml of 0.05 M sodium phosphate buffer (pH 7.0) and buffer containing H_2O_2 (0.02 and 0.08% w/v). The H_2O_2 solutions were prepared by diluting a stock solution (Sigma), standardized by using the molar extinction coefficient (ε_{240}) value of 43.6 1 mol^{-1} cm^{-1}. Immediately and after incubation for 60, 120 and 180 minutes at 37°C, serial dilutions were made in sterile 0.05 M sodium phosphate buffer (pH 7.0) and viability (in triplicate) was determined on Middlebrook 7H10 agar medium (BBL Microbiology Systems) containing 0.44% (v/v) glycerol, 0.2% (v/v) dextrose and 10% (v/v) OA enrichment. The media were incubated at 30°C and colonies counted after approximately 10 - 15 days.

RESULTS AND DISCUSSION

Catalase activity in extracts of M. avium were significantly differerent from measurements using the semi-quantitative test (Table 1). Strains Susook and 13528-1079 have similar crude extract activities (42.9 $\pm$ 4.4 and 42.0 $\pm$ 5.5 U/mg.) but widely different semi-quantitative catalase values (16.1 $\pm$ 1 and 50 $\pm$ 10 mm) Strains TMC 724 and 4443-1237 also gave similar disagreements between measurements. Because M. avium strains do not produce any extracellular catalase activity (Chapter 2), these differences in semi-quantitative catalase possibly reflect differences in the permeability of these organisms to H_2O_2.

In the study, cells of M. avium strains were exposed to 0.02 and 0.08% H_2O_2 and viability was determined initially and after 60, 120 and 180 minutes of incubation at 37°C. None of the M. avium strains tested were very susceptible to 0.02% H_2O_2 (Figure 1). Strains Susook and 13528-1079 were the most susceptible with 43% and 45% of cells still viable. The high degree of H_2O_2 tolerance of M. avium strains in this study is similar to values reported for M. intracellulare by Gangadharam and Pratt (10). This would be expected since both species are biochemically (21) and genetically related (2). It is also possible that some of the M. intracellulare strains that were studied by Gangadharam and Pratt (10) were actually M. avium strains, because the DNA-DNA hybridization studies by Baess (2) showed that several widely used strains of M. intracellulare actually belonged to the M. avium homology group.

H_2O_2 at a concentration of 0.08% was much more bactericidal and the M. avium strains fell into at least two groups based on their susceptibility (Figure 2). Strains V3435/77, TMC 724 and TMC 721 had very similar survival curves, (28-30% survival after 180 minutes of exposure). The low semi-quantitative

catalase activity of strains TMC 724 and TMC 721 (Table 1) suggest that these organisms may be relatively impermeable to H_2O_2. This would therefore contribute to their H_2O_2 resistance. Strain V3435/77, which was equally resistant to H_2O_2 (Figure 2), has a much higher semi-quantitative catalase activity (Table 1). It might therefore be expected to have a higher degree of H_2O_2 permeability. However, in this case, the high crude extract catalase activity (62.5 U/mg) may provide resistance to peroxide.

The remaining three strains of M. avium tested, were much more susceptible to 0.08% H_2O_2 (Figure 2). Strain Susook was most susceptible with only 0.1% survival after 180 minutes, followed by strain 13528-1079 (1.0% survival) and strain 4443-1237 (3.0% survival). The reason for the greater susceptibility of these strains over the previously mentioned group of M. avium strains may be partly due to greater H_2O_2 permeability as indicated by high semi-quantitative catalase measurements (Table 1). The greater H_2O_2 permeability of these strains, combined with their moderate catalase activities of extracts, in contrast to high permeability and high extract catalase activity of H_2O_2 resistant strain V3435/77 (Table 1 and Figure 2), may explain their susceptibility.

Permeability however does not seem to be the only factor contributing to differences in H_2O_2 susceptibility. Using semi-quantitative catalase activity as an indicator of permeability to H_2O_2, strain Susook should be less permeable to H_2O_2 than strain 13528-1079 and therefore less susceptible to its bactericidal effect. Both had equal crude extract catalase activities (Table 1). However, strain Susook was more susceptible to H_2O_2 than strain 13528-1079 (Figure 2).

A factor which may effect cell permeability is the composition of the cell surface of these bacteria (25). David (7) suggested that the lipid structure of

the cell wall outer layer may result in the multiple-drug resistance of M. avium by nonspecific exclusion of drugs. Lipid content has also been shown to play a major role in virulence of M. tuberculosis (11) and Jacket et al. (16) suggested that unsaturated lipids might provide protection against H_2O_2 by offering a substrate for harmless peroxidation reactions. It may be possible to increase the permeability of MAIS cells by growing them in the presence of Tween 80 for Mizuguchi et al. (22) and Hui et al. (14) found that Tween 80-grown M. intracellulare cells were more susceptible to antibiotics than those grown without Tween 80.

A study of Escherichia coli indicated that culture medium, growth phase and temperature play important roles in H_2O_2 sensitivity (30). In this study, The M. avium strains were grown in the same culture medium, at the same incubation temperature and harvested at the same growth stage. These factors therefore, should not have biased any comparisons between strains. Another factor which may effect mycobacterial H_2O_2 susceptibility is cell clumping, which occurs with most MAIS strains.

To identify and prove which factors are most influential to in vitro H_2O_2 susceptibility of M. avium catalase-negative and permeable mutants are required. Studying the in vitro susceptibility of mutant clones of a single strain, which differed in one variable (for example; specific catalase activity, semi-quantitative catalase, lipid content and cell clumping) might lead to more specific conclusions.

Table 1. Catalase activity of $\underline{M}$. $\underline{avium}$; specific activity and semi-quantitative measurement.

Strain	Sp. Act.[a] (U/mg $\pm$ S.D.)	Semi-Quantitative (mm of foam $\pm$ S.D.)
V3435/77[b]	62.5 $\pm$ 13.4	42 $\pm$ 6
TMC 724[c]	32.7 $\pm$ 1.3	4 $\pm$ 2
TMC 721	24.0 $\pm$ 14.9	6 $\pm$ 0
4443-1237[b]	34.5 $\pm$ 3.2	38 $\pm$ 5
13528-1079	42.0 $\pm$ 5.5	50 $\pm$ 10
Susook[b]	42.9 $\pm$ 4.4	16 $\pm$ 1

[a] Measured by the method of Beers and Sizer (4). Data from Chapter 2.

[b] Biochemically classified as $\underline{M}$. $\underline{intracellulare}$. DNA homology indicates $\underline{M}$. $\underline{avium}$ (2).

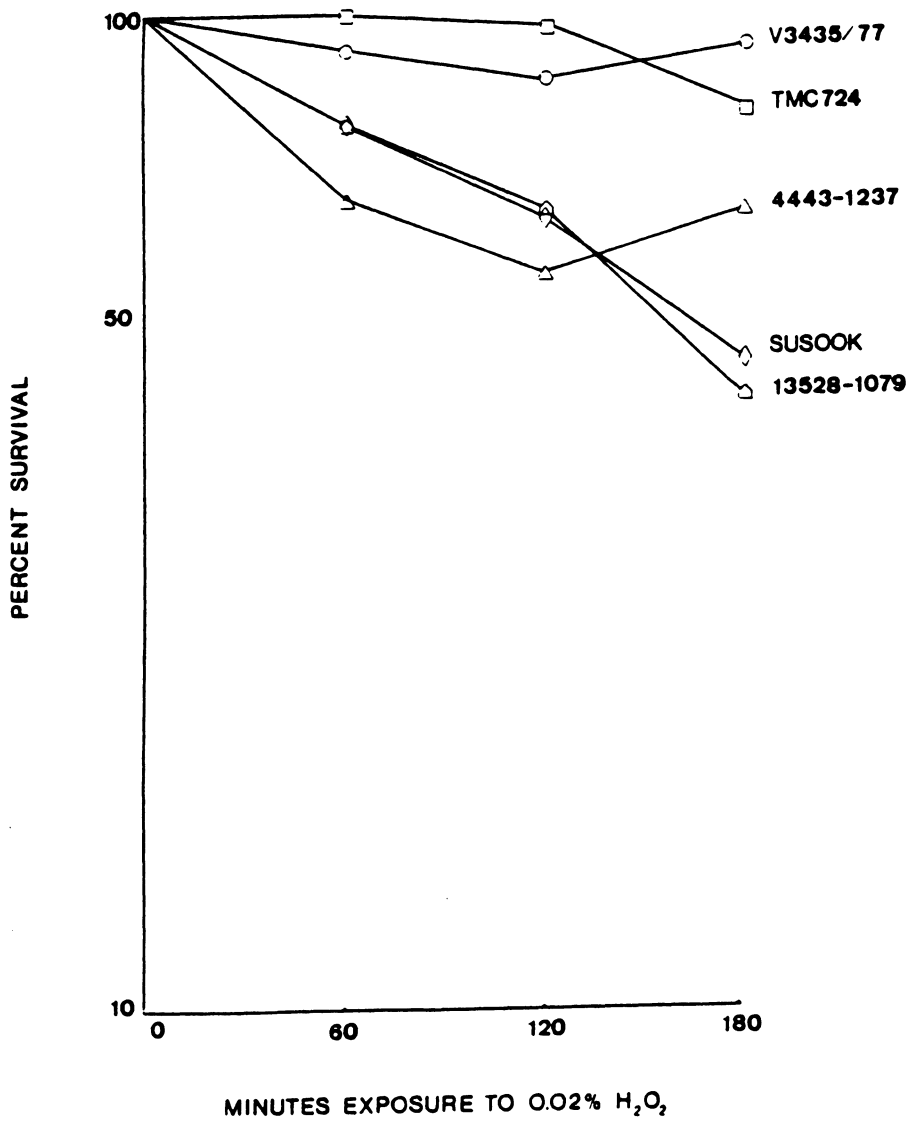[c] Type strain.

Figure 1. Effect of 0.02% hydrogen peroxide on the viability of <u>M</u>. <u>avium</u> V3435/77, TMC 724, 4443-1237, Susook, and 13528-1079.
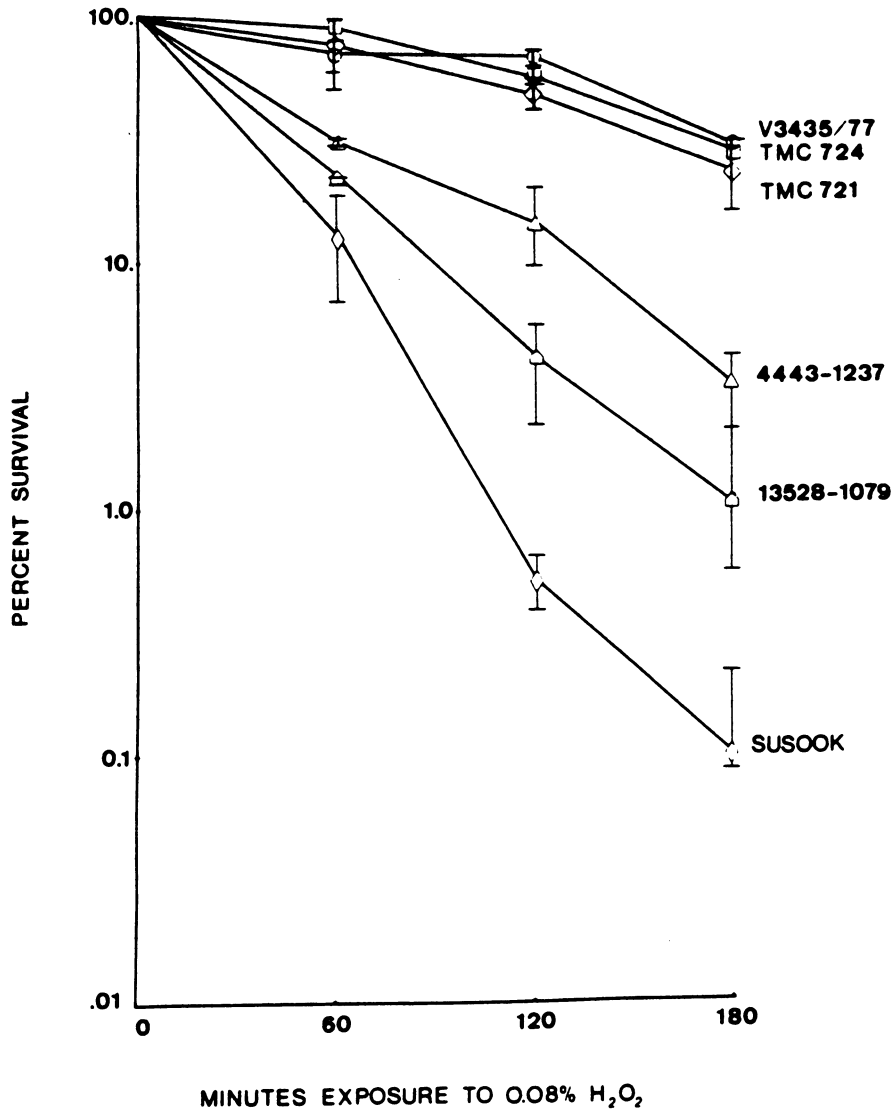
Figure 2. Effect of 0.08% hydrogen peroxide on the viability of M. avium V3435/77, TMC 724, TMC 721, 4443-1237, 13528-1079, and Susook. Mean ± range.

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Chapter 4

Superoxide Dismutase Activity

of

Mycobacterium avium, M. intracellulare and M. scrofulaceum.

INTRODUCTION

Superoxide dismutase (EC 1.15.11), which catalyzes the dismutation of the superoxide radical to hydrogen peroxide and molecular oxygen, has been found in a number of bacterial species (8, 24, 30, 35), since it was first purified from bovine erythrocytes by McCord and Fridovich (28). Because the generation of the superoxide radical is a normal product of the univalent reduction of molecular oxygen, superoxide dismutases are thought to be the primary defense against its potential cytotoxicity (14, 15). A study by Britton et al. (8) revealed that 17% of oxygen consumed by extracts of Streptococcus faecalis, which had their superoxide dismutase activity suppressed by the addition of specific antibody, was associated with the production of superoxide radicals. The observation that induced levels of superoxide dismutase correlate with greater resistance of bacteria to the toxic effects of hyperbaric oxygen (31) and superoxide generating compounds, such as plumbagin (12), supports the biological significance of this enzyme with respect to oxygen toxicity.

There are three distinct types of superoxide dismutases which can be distinguished by their metal content. Most procaryotes produce a superoxide dismutase with either iron or manganese as the prosthetic group, while eucaryotes have a copper-zinc cytoplasmic enzyme and a manganese-containing mitochondrial enzyme (5). Exceptions, within the procaryotes, include the copper-zinc superoxide dismutase producing bacteria; Photobacterium leiognathi (33), Pseudomonas diminuta, and P. maltophilia (34).

Within the genus Mycobacterium, superoxide dismutase has been characterized for a few species. These include Mycobacterium tuberculosis (25),

M. phlei (9), M. smegmatis (26), M. lepraemurium (21), and M. leprae (36). Currently there is no information on superoxide dismutase activity from the M. avium, M. intracellulare, and M. scrofulaceum (MAIS) group. This group of organisms, whose origin is environmental (37), have been reported with increasing frequency as the cause of human disease (18, 32). Most strikingly is the fact that a significant proportion of individuals suffering from acquired immunodeficiency syndrome (AIDS) have disseminated M. avium-intracellulare infection (38, 40).

Since MAIS organisms are capable of surviving and multiplying within the host lung (37) while exposed to a high partial pressure of oxygen and are capable of surviving the oxidative burst of activated macrophage (16), superoxide dismutase activity may be important to their pathogenicity. As environmental pathogens, MAIS organisms may be exposed to photochemically generated superoxide radicals in surface waters exposed to sunlight (10), and superoxide dismutase activity may aid in their survival. The study of MAIS superoxide dismutase activity is the subject of this report.

MATERIALS AND METHODS

Bacterial strains. Strains of mycobacteria used in this study (Table 1) were obtained from the National Jewish Hospital, Denver, Colorado and from the Statens Seruminstitut, Copenhagen, Denmark. Species assignments in Table 1 are based on DNA:DNA hybridization results (4, 22). Streptococcus lactis ATCC 11454 and Alcaligenes faecalis (unclassified) were obtained from the VPI microbiology department stock collection.

Growth of Cells and Preparation of Crude Extracts. Cell cultures and inocula of MAIS were grown in Middlebrook 7H9 medium (BBL Microbiology Systems, Cockeysville, MD.) supplemented with 0.44% (v/v) glycerol, 0.2% (v/v) dextrose and 10% (v/v) oleic acid-albumin (OA) enrichment. The latter was prepared as previously described (Chapter 2).

The MAIS inocula were grown in 16x150 mm screw-capped tubes containing 10 ml of the above medium at 37°C for approximately 9 days and stored for use at 4°C for a maximum of 2 months. MAIS cells were inoculated (1% v/v) into 1 liter screw capped flasks containing 300 ml of the enriched Middlebrook 7H9 medium and incubated with caps loose for 12 days at 37°C. Flasks were shaken by hand daily to resuspend the cells and aerate the medium. Cultures of S. lactis and A. faecalis were grown in 1 liter screw-capped flasks containing 300 ml of brain heart infusion broth (Difco laboratories, Detroit, MI.) on a rotary platform shaker for 18 hours (8). Following growth the cultures were streaked to confirm purity and 300 ml of the log-phase cells were harvested by centrifugation at 10,000 x g for 10 minutes, washed once in 100 ml of 0.05 M

potassium phosphate buffer, pH 7.8, containing 1 mM EDTA, and resuspended in a final volume of 10 ml of this buffer. Cells were broken by one passage through a cold French pressure cell (Aminco J4-3398A American Instument Co., Silver Spring, MD.) at 18,000 to 20,000 psi. The crude extracts were clarified by centrifugation at 23,000 x g for 30 minutes and the supernatants retained for enzyme assays.

Extracts used for superoxide dismutase inhibition studies, to indirectly determine metalloenzyme type, were dialysed (12,000-14,000 mw cutoff, Spectrapor membrane tubing, McGaw Park, IL.) for 24 hours at 4°C against 0.05 M potassium phosphate buffer, pH 7.8, containing 1 mM EDTA. The extracts were then centrifuged at 23,000 x g for 10 minutes to remove any precipitates.

Cultures for determination of extracellular superoxide dismutase activity were grown in 1 liter screw-capped flasks as previously described with the exception that OA enrichment was omitted and length of incubation was increased to 21 days. After centrifugation at 10,000 x g for 10 minutes, to remove cells, 300 ml of medium was concentrated to 10 ml by ultrafiltration through a PM-10 membrane (Amicon, Danvers MA.). The concentrate was then dialyzed for 24 hours at 4°C against 0.05M sodium phosphate buffer, pH 7.0 and centrifuged at 23,000 x g for 10 minutes.

Assay of Superoxide dismutase Activity. Superoxide dismutase was measured by the method of McCord and Fridovich (28). The amount of superoxide dismutase required to inhibit the rate of reduction of cytochrome c by 50% was defined as 1 unit of activity. KCN to a final concentration of 5×10^{-5} M was added to the assay system to inhibit any cytochrome oxidase which might be present in the

extracts (11). Protein was determined by the method of Lowry et al. (27) with bovine serum albumin (fraction V) as the standard.

Polyacrylamide Gel Electrophoresis (PAGE). Samples of crude cell-free extracts were examined for bands of superoxide dismutase activity in polyacrylamide slab gels. Electrophoresis was carried out as described in Chapter 2. The gels were stained for superoxide dismutase activity by the nitroblue tetrazolium method as described by Beauchamp and Fridovich (7).

Inhibition of Superoxide Dismutase Activity. The effect of KCN (1 mM final conc.) and NaN_3 (1, 5, and 10 mM final conc.) on superoxide dismutase activity were determined by the addition of either directly to the assay mixture. At these concentrations neither azide or cyanide inhibited the reduction of cytochrome c.

The effect of H_2O_2 was determined by incubation of the cell free extracts containing 5 mM H_2O_2 and 5 mM KCN at 22°C. The KCN was added to prevent the degradation of the added H_2O_2 by the catalase activity of the extracts. Following different periods of incubation, a 0.1 ml sample was removed and assayed for superoxide dismutase activity. Correction for the slight inhibition by H_2O_2 on cytochrome c reduction was made by addition of a slightly greater amount of xathine oxidase to the assay mixture.

RESULTS

<u>MAIS Superoxide Dismutase Activity</u>. The specific activity of cell-free extracts of representative strains of MAIS is shown in Table 1. <u>M</u>. <u>avium</u> strains had a mean superoxide dismutase activity of 27.6 $\pm$ 6.7 units/mg (mean $\pm$ standard deviation), <u>M</u>. <u>intracellulare</u> strains had a mean of 22.9 $\pm$ 4.5 units/mg, and the mean superoxide dismutase activity of the <u>M</u>. <u>scrofulaceum</u> strains was 16.2 $\pm$ 4.8 units/mg.

The superoxide dismutase activity was stable under refrigeration for at least 7 days, but was lost upon freezing extracts at 0°C. Extracts which were dialyzed prior to freezing retained their activity.

<u>Electrophoresis of MAIS Superoxide Dismutase</u>. Electrophoresis of crude extracts demonstrated a single band of superoxide dismutase activity for each of the MAIS strains tested (Figure 1). There were, however, differences in mobility. <u>M</u>. <u>avium</u> strains E 46941/76 and Susook, <u>M</u> <u>intracellulare</u> strains TMC 1406 and ATCC 25169 and <u>M</u>. <u>scrofulaceum</u> strain TMC 1323 all had an activity band of identical mobility (Figure 1, lanes 1-5), while the superoxide dismutase of <u>M</u>. <u>scrofulaceum</u> strain ATCC 19073 migrated further (Figure 1, lane 6). Of six <u>M</u>. <u>avium</u> and four <u>M</u>. <u>intracellulare</u> strains examined, all had identical mobility. Of the five <u>M</u>. <u>scrofulaceum</u> strains, two (TMC 1312 and TMC 1323) had superoxide dismutase activity which migrated as did that of <u>M</u>. <u>avium</u> and <u>M</u>. <u>intracellulare</u> and three strains (TMC 1306, TMC 1302, and ATCC 19073) had the faster migrating superoxide dismutase activity. The differences in mobility of the <u>M</u>.

scrofulaceum superoxide dismutases were not due to contamination by M. avium or M. intracellulare, as evidenced by streak plating.

Characteristics of MAIS Superoxide Dismutase Activity. The effect of various inhibitors on MAIS superoxide dismutase activity is shown in Table 2. Extracts from bacterial species of known metallotype have also been tested for comparison. All of the M. avium and M. intracellulare strains studied had superoxide dismutase activities which were moderately susceptible to 5 mM H_2O_2 and 1 to 10 mM NaN_3. The superoxide dismutase from M. scrofulaceum strains ATCC 19073, TMC 1302, and TMC 1306 were completely inactivated after 5 minutes of incubation with 5 mM H_2O_2 and exhibited greater sensitivity to 10 mM NaN_3, while M. scrofulaceum strains TMC 1312 and TMC 1323 were more resistant to both treatments. KCN (1mM) did not inhibit any of the MAIS superoxide dismutase activities.

Extracellular Superoxide Dismutase Activity. Superoxide dismutase activity was found in the concentrated medium after growth and removal of the cells. The activity of the concentrates for M. avium strains 13528-1079 and V3435/77 were 0.8 and 0.7 units/mg protein. M. intracelllare strains Manten 157 and Darden had slightly higher specific extracellular superoxide dismutase activities of 1.6 and 1.5 units/mg protein. M. scrofulaceum strains TMC 19073 and TMC 1323 also had measurable activities of 1.0 and 1.5 units/mg protein. Examination of concentrated medium by polyacrylamide gel electrophoresis demonstrated single bands of superoxide dismutase activity which correlated in mobility with activity of crude extracts. Assays of concentrated medium from M.

<u>scrofulaceum</u> strains TMC 1306 and TMC 1312 failed to demonstate any detectable superoxide dismutase activity (less than 0.3 units/mg protein.)

Table 1. Superoxide Dismutase activity of MAIS strains

Species/Strain	Total Assays	Superoxide[a] Dismutase
M. avium		
TMC 724	2	35.2 + 4.6
TMC 706	4	29.9 + 3.1
13528-1079	4	27.1 + 6.2
TMC 701	2	27.7 + 4.1
TMC 721	2	14.6 + 4.4
Wild strain E 46941/76	4	34.3 + 2.3
Susook	3	21.5 + 1.8
J 1868	2	25.3 + 2.1
Wild strain V 3435/77	2	32.4 + 7.4
4443-1237	2	22.4 + 2.9
Average + S.D.[b]	27	27.6 + 6.7
M. intracellulare		
TMC 1403	2	20.0 + 1.3
TMC 1406	4	25.8 + 4.2
Manten 157	2	26.3 + 4.0
TMC 1405	4	25.5 + 6.7
ATCC 25169	4	20.6 + 4.0
Darden	2	21.5 + 2.2
Mark Robert	2	20.6 + 1.8
2219, Altman	2	19.8 + 1.4
Average + S.D.	22	22.9 + 4.5
M. scrofulaceum		
TMC 1312	4	22.1 + 3.8
TMC 1306	3	18.3 + 1.5
TMC 1302	3	14.6 + 3.5
TMC 1323	4	11.6 + 2.9
ATCC 19073	4	14.3 + 3.6
Average + S.D.	18	16.2 + 4.8

[a] Mean units/mg. protein + standard deviation.

[b] Calculated from individual values for all strains and replicates.

Table 2. MAIS superoxide dismutase inactivation.

Species/Strain	Sp act (U/mg)	Inactivation by 5mM H_2O_2 (%)			Inactivation by NaN_3(%)			Inactivation by 1mM KCN (%)
		1'	5'	15'	1mM	5mM	10mM	
M. avium								
E46941/76	33.5	9	9	22	9	13	34	0
Sussok	23.5	35	35	35	15	26	32	-8
M. intracellulare								
TMC 1406	29.8	32	32	44	35	23	40	9
ATCC 25169	22.6	14	38	51	26	16	35	-31
M. scrofulaceum								
TMC 1323	11.6	33	44	44	6	17	22	-45
TMC 1312	17.5	57	57	81	14	14	39	-24
ATCC 19073	17.3	67	100	100	34	47	62	-25
TMC 1302	16.8	81	100	100	16	30	46	-22
TMC 1306	16.9	54	100	100	14	38	48	0
Alcaligenes faecalis								
Fe SOD[a]	14.3	ND	96	ND	0	31	48	5
Bacteroides fragilus VPI 2393								
Fe SOD[b]	810[d]	28	68	91	74	95	95	-23
Streptococcus lactis ATCC 11454								
Mn SOD[c]	20.3	ND	12	12	ND	ND	19	9

[a] Fe SOD, iron-containing superoxide dismutase. Reference 8.

[b] Purified by E. M. Gregory. Reference 31.

[c] Mn SOD, Manganese-containing superoxide dismutase. Reference 8.

[d] Units/ml.

Figure 1.   Polyacrylamide gel electrophoresis of MAIS cell-free extracts stained for superoxide dismutase activity.   Lanes: (1) M. avium strain E 46941/76; (2) M. avium strain Susook; (3) M. intracellulare strain TMC 1406; (4) M. intracellulare strain ATCC 25169; (5) M. scrofulaceum strain TMC 1323; (6) M. scrofulaceum strain ATCC 19073.

DISCUSSION

The average superoxide dismutase activities of MAIS cell-free extracts (Table 1) were slightly higher than most other species of mycobacteria. These include M. tuberculosis, 11.0 U/mg (25); M. smegmatis strain TAKEO, 12.0 U/mg (26); M. leprae, 0.17 U/mg (36); and M. phlei, 5.9 U/mg of protein(36). The superoxide dismutase activity of M. lepraemurium seems to vary, depending on whether the organism is grown in vivo or in vitro. Ichihara et al. (21) reported that the cell-free extract of M. lepraemurium grown in vitro (1% Ogawa egg yolk medium) had a specific activity of 180 U/mg, while Wheeler and Gregory (36) reported that M. lepraemurium grown in vivo (mice) had only 14.3 U/mg. This disparity (possibly due to differences in culture conditions) is of importance because of the DNA-relatedness of M. lepraemurium and M. avium (22).

Electrophoresis of crude, cell-free extracts demonstrated a single band of superoxide dismutase activity of all MAIS strains examined. There were, however, differences in mobility (Figure 1). All M. avium and M. intracellulare and two of five M. scrofulaceum reference strains tested had a superoxide dismutase activity band of identical mobility (Figure 1, lanes 1-5; slow migrating band). Three of the five M. scrofulaceum strains had a single, faster migrating superoxide dismutase activity band (Figure 1, lane 6).

Superoxide dismutases have been differentiated into three classes based on the type of metal ion that predominates as the prosthetic group. These classes are: copper-zinc-, manganese-, and iron-containing superoxide dismutases. Differentiation is possible indirectly by studies of inactivation and inhibition of the enzyme activity in crude, cell-free extracts. The copper-zinc superoxide

dismutases are inhibited by 1 mM KCN, while the manganese and iron forms are not (20). Irreversible inhibition of copper-zinc (19) and iron-containing (1) superoxide dismutases occurs in the presence of 5 mM H_2O_2, which does not effect the manganese-containing enzyme. Addition of 10 mM NaN_3 will inhibit all three superoxide dismutases, but to different degrees. The iron-containing superoxide dismutase is most sensitive, followed by the manganese and then the copper-zinc form (29).

The data presented in Table 2 suggest that MAIS superoxide dismutases possess characteristics of both manganese- and iron-containing enzymes. An iron-containing superoxide dismutase should have been inactivated completely by incubation with 5 mM H_2O_2, as evident with the control strains (A. faecalis 96% inhibited, B. fragilis 91% inhibited), while a manganese-containing superoxide dismutase should have been resistant (S. lactis 12% inhibited). The superoxide dismutase activities of extracts from M. avium and M. intracellulare strains (Table 2) were more intermediate in their resistance (22% - 51% inhibition after exposure to 5 mM H_2O_2 for 15 minutes). Superoxide dismutase activity of the M. scrofulaceum strains, with the exception of TMC 1323, were even more sensitive to H_2O_2. M. scrofulaceum strains ATCC 19073, TMC 1302 and TMC 1306 were 100% inhibited after only 5 minutes of incubation with 5 mM H_2O_2 and the superoxide dismutase from TMC 1312 was 81% inhibited after 15 minutes. The superoxide dismutases of the M. scrofulaceum stains, which exhibited greater sensitivity to H_2O_2 were also more sensitive to 10 mM NaN_3. These characteristics are more indicative of iron-containing superoxide dismutases.

A similar pattern of inhibition of MAIS superoxide dismutase activities by H_2O_2 was also apparent in polyacrylamide gels (data not shown). When 5 mM H_2O_2 and 1 mM KCN were added to the riboflavin and nitroblue tetrazolium solutions (solutions for assaying superoxide dismutase activity in polyacrylamide gels; 6) the activity bands of M. scrofulaceum strains TMC 1302 and TMC 1312 were completely inhibited, while M. scrofulaceum strain TMC 1323 still displayed a faint band. The superoxide dismutase activity bands of M. avium strain Susook and M. intracellulare strain TMC 1406 were inhibited approximately 50%, but still clearly visable. By comparison A. faecalis, which displayed a single superoxide dismutase activity band, was completely inhibited and the superoxide dismutase band of S. lactis was not significantly inhibited at all.

Interestingly, all of the reference strains tested which displayed the slower migrating band were also intermediate in their sensitivity to H_2O_2 and NaN_3, while the superoxide dismutase activity from the M. scrofulaceum strains with the faster migrating superoxide dismutase band were much more sensitive (Table 2, Fig. 1). The slight differences in mobility may therefore correlate with differences in metal composition.

Inhibition studies on superoxide dismutase in crude extracts from Nocardia asteroides, also an intracellular pathogen, gave results similar to those reported here for the slow migrating form. Treatment with 20 mM NaN_3 and 5 mM H_2O_2 resulted in 41% and 40% inhibition, respectively (6). Analysis by atomic absorption spectroscopy subsequently detected almost equimolar concentrations of Mn, Fe, and Zn (6). M. phlei is another organism which has been shown to contain relatively large proportions of all three metals (9). Atomic absorption

spectroscopy measured 1.2, 1.7, and 0.8 gram atoms/mol enzyme of Fe, Mn, and Zn, respectively (9). The exact metal composition of MAIS superoxide dismutases will require similar analysis by atomic absorption.

Studies of superoxide dismutase produced by other species of mycobacteria have reported that M. leprae (36), M. smegmatis strain TAKEO (26) both produce a manganese-containing superoxide dismutase, while M tuberculosis produces an iron - containing superoxide dismutase (25). Atomic absorption analysis of superoxide dismutase from M. lepraemurium (21) has shown that the enzyme contains manganese (1.3 gram atoms/mol) and a small but significant amount of iron (0.3 gram atoms/mol). Since M. lepraemurium and M. avium have a high degree of DNA-relatedness (22) atomic absorption spectroscopy of M. avium superoxide dismutase might be expected to give similar results.

Examination of concentrated medium from some MAIS cultures demonstrated the presence of superoxide dismutase activity. Polyacrylamide gel electrophoresis of concentrated medium in which these strains were grown demonstated a single band of superoxide dismutase activity which correlated in mobliity with activity of crude extracts. Concentrated medium in which M. scrofulaceum strain TMC 1306 or TMC 1312 was grown failed to exhibit any measurable activity by either the cytochrome c reduction assay or polyacrylamide gel electrophoresis. The fact that all of the M. scrofulaceum strains did not exhibit extracellular superoxide dismutase activity, even though cultured under identical conditions, suggests that the activity present in the concentrated medium of other MAIS strains was not the result of cell lysis.

Superoxide dismutase activity of concentrated culture medium has been reported for Mycobacterium tuberculosis (25) and Nocardia asteroides (6); both

intracellular pathogens. Immunofluorescent staining of live cells of N. asteroides indicated the association of superoxide dismutase with the outer cell envelope (6). Conversely, examination of concentrated culture media of rarely pathogenic M. smegmatis (25) and nonpathogenic strains of N. asteroides (6) failed to detect any extracellular superoxide dismutase activity.

It has been suggested that the secretion of superoxide dismutase by Nocardia asteroides, as well as its association with the outer cell envelope, could provide protection against killing by superoxide radicals (6) which are produced during active phagocytosis (2, 3) and Filice et al. (13) have shown that N. asteroides is resistant to the phagocytic attack of human neutrophils and monocytes. The fact that exogenously added superoxide dismutase has been shown to provide protection to bacteria against phagocytic attack (23, 39) also illustrates the importance of superoxide dismutase.

A study by Gangadharam et al. (16) has demonstrated that alveolar and peritoneal mouse macrophages readily phagocytosed M. intracellulare in vitro, but could not indefinitely prevent growth of the ingested bacilli regardless of their state of activation. The production and secretion of superoxide dismutase by certain MAIS strains may therefore serve a protective function against phagocytic killing. It would be interesting to determine whether superoxide dismutase activity is associated with the outer cell surface of MAIS organisms, as demonstrated with N. asteroides. This association could provide protection to MAIS present in aquatic habitats (17) where photochemically generated superoxide radicals may be present (10).

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CHAPTER 5

SUMMARY

Catalase and superoxide dismutase, but not peroxidase activity was detected in crude, cell-free extracts of all MAIS strains studied. The lack of peroxidase activity is in accord with the observations of Tirunaryanan and Vischer (12), who did not detect peroxidase activity in M. avium.

Catalase activity of MAIS was determined with whole cells as well as crude, cell-free extracts. The whole cell method was a semi-quantatative estimation of catalase activity in which bubble production was measured, after a H_2O_2:Tween 80 solution was added to the culture (8). A bubble height of less than 45 mm is considered catalase negative and anything greater than 45 mm is considered catalase positive (8). With this method most M. avium and M. intracellulare strains were catalase negative, with the exception of M. avium strains V3435/77 and 13525-1079 which were catalase positive. All M. scrofulaceum strains were catalase positive with bubble heights well above 45 mm.

The measurement of catalase activity in crude, cell-free extracts by the Beers and Sizer method (4) demonstrated that M. avium and M. intracellulare strains had very similar catalase activities with average values of 34.7 and 35.5 units/mg of protein, respectively. The catalase activity of the M. scrofulaceum strains was much higher with an average value of 134.8 units/mg.

By heating cell-free extracts of MAIS reference strains at 53°C and measuring residual catalase activity after 50 minutes of incubation, significant differences between all three species were observed. M. intracellulare catalase was very sensitive to heat treatment and retained only 14.1% activity. By contrast, M. avium catalase was intermediate in susceptibility with 53.3%

activity remaining after 50 minutes at 53°C, while M. scrofulaceum catalase was very resistant and retained 82.2% activity.

Visualization of catalase activity on polyacrylamide gels was accomplished by the diaminobenzidine method (6). With this method crude extract from M. avium and M. intracellulare strains consistantly displayed two bands of catalase activity (band 2 being of greater area), while extracts from M. scrofulaceum strains displayed four bands. Bands 1 and 2 of all three MAIS species had identical mobility rates, while bands 3 (a minor band) and 4 (equal in area to band 2) of M. scrofulaceum strains migrated further. Other researchers who stained for mycobacterial catalase activity using the H_2O_2-KI system had inconclusive results due to poor resolution (10, 13).

Examination of heat-treated extracts (50' @ 53°C) by polyacrylamide gel electrophoresis, demonstrated that bands 1 and 2 of all M. intracellulare and M. scrofulaceum strains and band 2 of all M. avium strains tested were sensitive to heat inactivation. Activity band 1 of all M. avium strains increased in intensity as if being activated. The heat activation seen in band 1 could explain the intermediate resistance seen with M. avium catalase using the Beers and Sizer method (4). Band 3 of M. scrofulaceum, which was only a minor band of catalase activity, displayed moderate resistance to heat inactivation. M. scrofulaceum band 4, which was very resistant, probably is responsible for the greater heat resistance of M. scrofulaceum over strains of M. avium and M. intracellulare.

In a further attempt to characterize the catalytic activities separated by electrophoresis, 3-amino-1,2,4-triazole (AT) resistance was measured. Bands 1 and 2 of all MAIS strains were resistant to AT inhibition. Band 3 of M. scrofulaceum catalase was AT sensitive and band 4 was moderately sensitive.

Mycobacteria have been reported to produce two different classes of catalase; T class (heat-labile, AT-resistant) and M class (heat-stable, AT-sensitive) (14). According to Wayne and Diaz (14), M. intracellulare and M. avium strains produce only T catalase, while M. scrofulaceum strains produce both T and M catalase. My results agree with this observation. Activity bands 1 and 2 in M. intracellulare and M. scrofulaceum and band 2 in M. avium are characteristic of T catalase, while bands 3 and 4 in the M. scrofulaceum strains are characteristic of M catalase. More work needs to be done to explain the apparent heat activation that occurs in activity band 1 of the M. avium strains.

Heat sensitivity of MAIS catalase is the first phenetic trait capable of speciating MAIS. Others may have failed to find species-specific traits because they may not have studied true representatives of each species.

In addition to high crude extract catalase activity, M. scrofulaceum, but not M. avium and M. intracellulare, demonstrated extracellular catalase activity. By removing cells after growth and concentrating the culture medium (30-fold), 1.8, 2.1 and 5.7 units/mg of protein of catalase activity was detected for M. scrofulaceum strains TMC 1312, TMC 1306 and TMC 1323, respectively. This catalase activity was inactivated by 1 mM KCN, indicating a traditional catalase protein. Electrophoresis of concentrated medium of M. scrofulaceum strain TMC 1323, demonstrated a single band of catalase activity which was identical in mobility to M. scrofulaceum band 4. The fact that only band 4 was present in the concentrated culture media, supports the conclusion that it is a true extracellular catalase enzyme and not simply the result of cell lysis.

Previous studies have indicated that catalase-positive strains of Mycobacterium tuberculosis (11) and M. intracellulare (7) have high resistance

to 0.02% H_2O_2. In this study catalase positive M. avium strains exhibited similar resistance (43 - 92% survival). H_2O_2 at a concentration of 0.08% was more bactericidal (than 0.02%) and the M. avium strains fell into two groups based on their susceptibility. Strains V3435/77, TMC 724 and TMC 721 had similar survival curves with 28-30% survival after 180 minutes of exposure. The second, less resistant group of M. avium strains (Susook, 13529-1079 and 4443-1237) had survival values of only 0.1-3.0%. The greater susceptibility of these strains could be correlated with greater permeability to H_2O_2. In general, strains which demonstrated greater catalase activity and less H_2O_2 permeability were more resistant to H_2O_2. It was evident however, from survival values of strains 13528-1079 and Susook, that other factors must also be involved. After exposure to 0.08% H_2O_2 for 180 minutes strain 13528-1079 displayed 1.0% survival, while strain Susook had 0.1% survival. These strains had equivalent catalase activities and strain 13528-1079 was much more permeable to H_2O_2. Based on permeability, strain 13528-1079 should have been more susceptible.

This is the first report of superoxide dismutase activity from MAIS organisms. M. avium strains had an average superoxide dismutase activity of 27.6 units/mg of protein, M. intracellulare had an average of 22.9 units/mg and the average superoxide dismutase activity of M. scrofulaceum strains was 16.2 units/mg. Polyacrylamide gel electrophoresis of crude extracts demonstrated a single band of superoxide dismutase activity for all MAIS strains examined. All M. avium and M. intracellulare and two of five M. scrofulaceum strains tested had a superoxide dismutase activity band of identical mobility, while extract from the remaining three M. scrofulaceum strains demonstrated a single, faster migrating superoxide dismutase activity band.

Superoxide dismutase activity was also detected in concentrated culture medium from all M. avium, M. intracellulare and two of four M. scrofulaceum strains tested. Examination of concentrated culture medium by polyacrylamide gel electrophoresis demonstrated single bands of superoxide dismutase activity which correlated in mobility with activity of crude extracts. Extracellular superoxide dismutase activity and cell surface associated superoxide dismutase activity, has been previously reported for pathogenic strains of M. tuberculosis (3, 9) and Nocardia asteroides (1, 2). In contrast, nonpathogenic strains of M. tuberculosis (3, 9) and N. asteroides (1, 2) lacked both extracellular- and cell surface-associated superoxide dismutase activity. It would be of interest to determine if the MAIS extracellular superoxide dismutase is surface associated as well. Surface associated superoxide dismutase activity together with intracellular superoxide dismutase and catalase activity and what seems to be a naturally high resistance to H_2O_2, would be of adaptive value in defense against O_2^-, H_2O_2 and $OH^{\cdot}$ generated in host macrophage and sunlight illuminated waters.

Inhibition of superoxide dismutase activity with KCN, H_2O_2 and NaN_3, suggested that manganese and iron may both be present in the enzyme of M. avium, M. intracellulare and two of five M. scrofulaceum strains. By contrast, the pattern of inhibition of superoxide dismutase activity of extracts from the remaining three M. scrofulaceum strains were characteristic of iron containing superoxide dismutase. The presence of more than one metal in a single superoxide dismutase enzyme is not a common phenomenon, but has been reported for M. phlei (5) and N. asteroides (1). The exact determination of MAIS

superoxide dismutase metal content will require purification and analysis by atomic absorption spectroscopy.

The major impetus for this research was the observation that the higher frequency of MAIS isolated from waters of the southeastern United States was due to the increased recovery of pigmented, catalase-producing strains. The data support the contention that catalase is a factor influencing H_2O_2 susceptibility. In addition, permeability of cells to H_2O_2 was also shown to be a factor. While studying the heat sensitivity of MAIS catalase, it was discovered that distinct, species-specific differences existed. This could prove to be a very useful method of species identification.

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