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Serological, Taxonomic, and Kinetic Studies of the T and M Classes of Mycobacterial Catalase

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Two types of catalase may be found in extracts of mycobacteria, the heat-labile T class and the heat-stable M class. The T-catalase is resistant to 3-amino-1,2,4-triazole and has a Michaelis constant in the range of 3.1 to 6.8 mM H_2O_2, whereas the M-catalase is inhibited by 3-amino-1,2,4-triazole and has a Michaelis constant in the range of 143 to 156 mM. Some species of mycobacteria produce only one class of catalase, and others produce both. Of the species studied, only *Mycobacterium terrae*, *M. triviale*, and *M. nonchromogenicum* failed to exhibit T-catalase, although all three of these species had M-catalase. Conversely, *M. tuberculosis*, *M. bovis*, *M. intracellulare*, *M. avium*, *M. gastri*, *M. marinum*, and *M. xenopi* yielded T-catalase but not M-catalase. Six species, *M. szulgai*, *M. simiae*, *M. kansasii*, *M. gordonae*, *M. scrofulaceum*, and *M. asiaticum*, produced both classes. The differences in resistance to heat and 3-amino-1,2,4-triazole were exploited in the development of methods for quantitative serological characterization of one class of catalase in the presence of the other. These techniques were used with three reference sera to produce a branching diagram of divergence of the T-catalases from 13 species of mycobacteria based on measurements of immunological distance. No T-catalase could be demonstrated in another three species. A first-stage study was also carried out with a single reference antiserum to M-catalase from *M. kansasii*. Representatives of nine mycobacterial species, including the three that produced no T-catalase, were characterized with this reference system, which tends to yield larger immunological distance values than the T-catalase system.

Mycobacteria produce two classes of catalase (EC 1.11.1.6), which can be distinguished from one another by their sensitivities to thermal inactivation. The original studies of these catalases were based on heating whole cells to 68°C for 20 min (9, 13, 16). Bartholomew detected a single, heat-labile catalase in zymograms of sonic extracts of *Mycobacterium gastri*, but found one heat-labile band and one heat-stable band in *M. smegmatis* (3). The stability of the latter was only apparent in extracts of whole cells that were heated before sonication; i.e., if the cell-free extracts themselves were heated to 68°C for 20 min, the activities of both *M. smegmatis* bands disappeared. It has since been reported that the difference in thermal stability of the two classes of mycobacterial catalase can be measured more precisely in cell-free extracts if the inactivation rates are determined at 53°C (14).

Catalases derived from *M. tuberculosis* and *M. bovis* have been reported to differ with respect to sensitivity to several inhibitors from the catalases of a number of other mycobacterial species and the catalase of bovine liver (2, 7). The most striking difference was in the relative sensitivity to the effects of 3-amino-1,2,4-triazole (AT) (7). The ability of whole mycobacterial cells to bind isonicotinic acid hydrazide and the loss of catalase after acquisition of resistance to high levels of isonicotinic acid hydrazide are both associated with the heat-labile class of catalase (16).

Throughout this report, the heat-labile class, which was first noted in *M. tuberculosis* (9, 13), is referred to as T-catalase, and the heat-stable catalase, which more closely resembles an enzyme derived from mammalian tissues, is referred to as M-catalase.

To date, T-catalases have been detected in extracts of at least eight species of mycobacteria, and all of these enzymes have been shown to be related to one another immunologically (14, 15). The M-catalases do not cross-react with antisera to the T-catalases. *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. avium*, *M. intracellulare*, and *M. gastri* produce T-catalases, but M-catalases have not been found in these species (14, 15). Both M- and T-catalases have been detected in *M. kansasii* and *M. scrofulaceum*. Until now, there have been no reports of species

of *Mycobacterium* which produce M-catalase exclusively. In species that produce both enzymes, the M-catalase tends to obscure the T-catalase, making it necessary to subject the crude cell extracts to several stages of purification before the T-catalase can be studied serologically.

The immunological relatedness of the T-catalases derived from nine species of mycobacteria has been described previously (14, 15). This relatedness was expressed in terms of immunological distance (ImD), which was calculated from the ratio of the amounts of specific antiserum required to remove standard amounts of heterologous and homologous catalases from cell extracts (5, 11, 14). The assay for the unprecipitated catalase used a concentration of substrate which yielded zero-order kinetics with T-catalase and permitted measurement of very small amounts of the enzyme. The attainment of saturation kinetics was unexpected, since catalases from other sources exhibit Michaelis constants (K_m) so high that these kinetics cannot be achieved (i.e., saturating concentrations of H_2O_2 would destroy the enzyme) (1).

Determinations of ImDs between T-catalases from pairs of mycobacteria which produce only this class of catalase have been performed readily by the methods described previously, in which the endpoint of the titration is based on an assay of the enzyme activity remaining in the supernatant. However, when extracts of cells that produce both classes of catalase are used or when very high concentrations of serum are required, which introduce intrinsic peroxide-destroying substances from the serum itself, the masking effects of these M-catalase products are amplified by the high concentration of H_2O_2 needed for the assay. It would be especially useful to be able to carry out a serological titration of either class of catalase in crude extracts of all mycobacteria without the need for extensive preliminary fractionation.

We undertook this investigation to clarify the kinetics of the two classes of mycobacterial catalase, to establish the reliability of alternative methods for the assay of the catalases in serological reaction products in order to avoid masking effects, and to extend our ImD studies to include additional reference systems.

MATERIALS AND METHODS

Bacterial strains. Strains of mycobacteria from our own collection (designated W) and from the Trudeau Mycobacterial Collection (designated TMC and provided through the courtesy of the United States-Japan Cooperative Medical Sciences Program) were used as sources of catalase.

Preparation of catalase. The methods for growing the cells, disrupting them, and separating the crude proteins have been described previously (15). With strains that contained only the T-catalase, the crude protein extracts were used directly for catalase assay. Some crude protein extracts of *M. kansasii* TMC 1201 and *M. scrofulaceum* TMC 1314 were further fractionated by ammonium sulfate precipitation. The fractions precipitating between 30 and 70% saturation were subjected to chloride gradient ion-exchange chromatography on diethylaminoethyl Sephadex (7). The fractionation was carried only to the point where the two classes of catalase were separated from one another but neither product was further separated from other proteins. Additional batches of extracts containing both classes were left unfractionated to test methods that were developed for serological characterization of one class of catalase in the presence of the other.

Assay of catalase activity. Two systems of assaying the catalase activities of the specimens were used in this study. The original system was based on a first-order reaction (7). The enzyme sample was allowed to react at 37°C for 2 min with 100 μg (2.94 μmol) of H_2O_2 in a total volume of 3 ml of 0.01 M phosphate buffer at pH 7.0. The addition of 1.5 mg of $TiCl_4$ in 1.5 ml of 9 N H_2SO_4 stopped the reaction and resulted in the formation of a colored complex with the residual H_2O_2. The optical absorbance at 410 nm (A_{410}) was determined, and the amount of residual substrate was calculated from a standard curve. The activity of the enzyme was expressed as the log of the ratio of the original concentration of H_2O_2 (A_o) to the final concentration of H_2O_2 (A_t); 1 U of enzyme activity was defined as the amount that destroyed 90% of the original substrate in 2 min (i.e., $\log A_o/A_t = 1.0$). The advantage of this method was that it could be used for both classes of mycobacterial catalase. The disadvantage was the lower limit of sensitivity (about 0.04 U of activity) (14). Accurate serological titrations required 10 times this amount, representing the product of 40 mg of moist mycobacterial cells for each tube in a series. Therefore, this method was used only to establish the units of activity in a given extract, and the following system was used in the serological titrations.

The second system was based on saturation kinetics and was adopted after it was noted that the heat-labile T-catalase exhibited saturation kinetics when the substrate concentration exceeded 7 mM (14). In this system the enzyme sample was allowed to react at 37°C for 5 min with 100 μg (2.94 μmol) of H_2O_2 in a total volume of 0.15 ml of a solution containing 2 mg of bovine albumin fraction V per ml in 0.1 M phosphate buffer at pH 7.5 (APB). Addition of 1.45 mg of $TiCl_4$ in 4.4 ml of 3 N H_2SO_4 stopped the reaction and resulted in the formation of a colored complex with the residual H_2O_2. Under these conditions, 0.05 first-order unit of T-catalase destroyed enough H_2O_2 to yield a decrease in A_{410} of 0.33 U and thus was sufficient for seroprecipitation studies in which zero-order kinetic calculations were used. As discussed below, the concentration of substrate used in this assay was not sufficient to saturate M-catalase, but it was possible to use the same technique (by simply substituting first-order kinetic calculations) to determine the activity in a sample of M-catalase. Therefore, we refer to this method as the routine catalase assay method, with the stipulation that calculations were based on saturation kinetics when T-catalase was studied and on first-order kinetics when M-catalase was under investigation.

To determine K_m values for mycobacterial catalases, the routine catalase assay method was modified slightly. Different volumes and substrate concentrations were used for the two enzyme classes in order to facilitate accurate location of the intercepts. The amount of H_2O_2 destroyed in 1 min represented the initial velocity of the reaction. The K_m values were determined from Lineweaver-Burk plots of the reciprocals of the initial velocities versus the reciprocal substrate concentrations in the ranges tested (1 to 6 mM for T-catalase and 40 to 200 mM for M-catalase).

Preparation of reference antisera. Antisera against T-catalase and M-catalase fractions of extracts of *M. kansasii* TMC 1201 were prepared in New Zealand white rabbits. A 15-U sample (0.3 mg of protein) of the T-catalase, which was recovered by gradient chloride diethylaminoethyl chromatography and was dissolved in 1.5 ml of phosphate-buffered saline, was mixed with 1 mg of immunoadjuvant peptide (*N*-acetyl-muramyl-L-alanyl-D-isoglutamine; Calbiochem-Behring) and 1.5 ml of Freund incomplete adjuvant (Difco Laboratories). Each of two rabbits received 0.5 ml of this emulsion subcutaneously and an additional 1.0 ml intradermally divided among 7 to 10 sites. Similarly, 122 U (3.8 mg of protein) of the M-catalase in 1.5 ml of buffer was mixed with immunoadjuvant peptide and Freund incomplete adjuvant and inoculated into two more rabbits. The rabbits were bled after 29 days, and the sera were used for the catalase titrations.

Serological titration. The simple supernatant method which we have used to establish ImD scores for preparations that contain only T-catalase has been described previously (15). Briefly, 100-μl aliquots of catalase were mixed with 100 μl of each of the serum dilutions in 400-μl polyethylene centrifuge tubes and incubated at 37°C for 1 h, followed by overnight incubation at 5°C. The tubes were centrifuged, and 100 μl of each supernatant was removed and analyzed for residual catalase by the routine catalase assay method. The calculation of binding capacities and ImD has been described previously (16).

Two methods were used for serological titration of T-catalase in the presence of M-catalase. One of these methods was based on an assay of the enzyme in the serological precipitate itself. A 50-μl amount of catalase was mixed with 50 μl of each serum dilution in a 10-ml conical centrifuge tube and incubated as described above; 5 ml of APB was then added to the reactants, and the preparation was centrifuged immediately. Each supernatant was decanted and discarded, and the tube was drained for 1 h in an inverted position. The precipitate was suspended in 100 μl of APB, and its catalase activity was determined by the routine catalase assay method.

The second method was based on the relatively specific inhibition of M-catalase but not T-catalase by high concentrations of AT. This method was performed exactly as the simple supernatant method was, with the addition of one step. Before the H_2O_2 substrate for the routine catalase was added, 10 μl of a 100-mg/ml suspension of AT in APB was added to the 100 μl of the antigen-antibody supernatant. This mixture was allowed to stand for 2 h at room temperature, and the H_2O_2 was then added as described above for the routine catalase assay. Thus, a much higher concentration of AT and a longer exposure to AT were used than in our previous study (7).

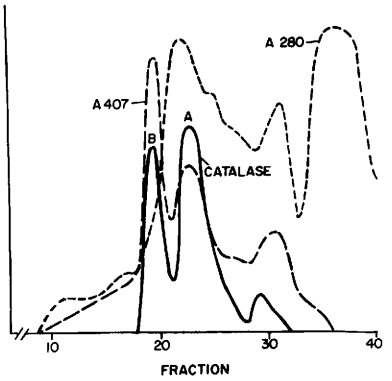

FIG. 1. Diethylaminoethyl Sephadex ion-exchange chromatography of a sonic extract of *M. kansasii*, showing the relative protein concentration (absorbance at 280 nm [A 280]), Soret band absorption (absorbance at 407 nm [A407]), and catalase activity. The major peaks representing the heat-labile T-catalase (peak A) and the heat-stable M-catalase (peak B) are identified.

For serotitrations of M-catalase, the extracts were first heated to 68°C for 60 s by dilution in prewarmed APB and then rapidly chilled in an ice bath. This inactivated the T-catalase, and serotitration of the M-catalase could then be carried out by the simple supernatant method; first-order calculations were used to determine the residual enzyme concentrations. In all cases, a high concentration of anti-M-catalase serum removed all of the activity, thus assuring that the heat had eliminated the T-catalase.

RESULTS

Separation and kinetic studies of the two catalase classes. Crude protein extracts of *M. kansasii* TMC 1201 and *M. scrofulaceum* TMC 1314 were subjected to ammonium sulfate precipitation and ion-exchange chromatography. Both of these preparations yielded two major peaks of catalase activity, which corresponded to peaks of optical absorbance at 407 nm (i.e., the Soret band). A small third peak was observed at the right of the elution profile of the *M. kansasii* extract (Fig. 1). Material from this peak exhibited kinetic behavior like that of M-catalase. This material was used to immunize a pair of rabbits and was found to be serologically identical to the material in peak B; i.e., it represented the same protein as the M-catalase in peak B, but it may have been in a different state of aggregation. Peak A corresponded to T-catalase.

Samples from each of the two major peaks from the ion-exchange column eluates of *M. kansasii* and *M. scrofulaceum*, as well as bovine liver catalase (Sigma Chemical Co., St. Louis, Mo.) and crude extracts containing only T-catalase from five other mycobacterial species, were subjected to kinetic study. The T-catalase

TABLE 1. K_m values of two classes of mycobacterial catalase

Source	Strain	K_m (mM) as related to stability at 53°C	
		T-catalase	M-catalase
M. tuberculosis	H37Rv	5.3 ± 1.8[a]	
M. tuberculosis	K9775	3.1 ± 0.5	
M. bovis BCG	TMC 1002	3.9 ± 1.1	
M. bovis BCG	TMC 1011	3.6 ± 0.6	
M. avium	TMC 716	5.0 ± 0.5	
M. intracellulare	TMC 1403	5.3 ± 0.3	
M. gastri	W417	4.2 ± 0.6	
M. kansasii	TMC 1201	6.8 ± 2.6	143 ± 19
M. scrofulaceum	TMC 1314	6.7 ± 0.6	156 ± 44
Bovine liver			121 ± 20

[a] Mean ± standard deviation.

products derived from representatives of seven species exhibited apparent K_m values ranging from 3.1 to 6.8 mM H_2O_2 (Table 1). The M-catalases from *M. kansasii* and *M. scrofulaceum* had apparent K_m values of approximately 150 mM H_2O_2, which were comparable to the value observed for bovine liver catalase.

The high K_m values observed for M-catalases made it clear that a zero-order assay for M-catalases could not be established. It had already been demonstrated that prolonged incubation (5 min) in the routine catalase assay increased the sensitivity of the assay without detracting from the linearity of the dose response of T-catalase (14). It was still possible that the same technique could yield a similar dose-response curve for M-catalase if first-order calculations were used to calculate enzyme concentrations. Therefore, a series of dilutions of M-catalase from *M. kansasii* was assayed by this technique, and the dose response was found to be linear (Fig. 2).

Estimation of T-catalase in the presence of M-catalase. Samples of *M. kansasii* TMC 1201 T-catalase that were separated chromatographically from the M-catalase component (0.05 U in 100 μl) were incubated for 2 h with graded amounts of AT ranging from 0.03 to 1.0 mg before the substrate for the routine assay was added. Similarly, samples of a crude extract containing both T- and M-catalases (total enzyme activity, 0.025 to 0.30 U) were also incubated with graded amounts of AT. The maximum amount of AT used (1.0 mg) effected only a 10% reduction in the activity of T-catalase (Fig. 3) but totally inactivated the M-catalase component in the mixtures when these mixtures contained as much as 0.2 U of mixed enzyme activity (or of chromatographically isolated M-catalase activity alone, as determined separately). The substrate used in the routine catalase assay yielded an A_{410} of 0.530 U, so a change in the A_{410} (ΔA_{410}) greater than this could not be measured, thus limiting the amount of enzyme that could be tested. Thus, within the range from 0.025 to 0.20 U of mixed enzyme activity, the use of AT at a concentration of 10 mg/ml permitted the quantitative estimation of T-catalase in the presence of M-catalase and demonstrated that the T-catalase class represented about one-third of the total catalase activity in the mixture (Table 2); that is, after treatment with AT, 1 U of mixture gave a

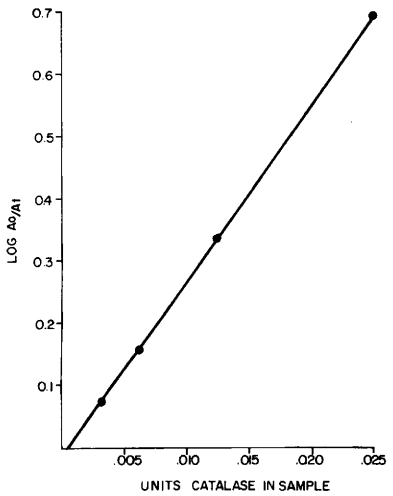

FIG. 2. Linearity of the dose response of M-catalase from *M. kansasii* TMC 1201 in the routine catalase assay when first-order calculations were used. A_o, A_{410} of the reaction product of the substrate in the absence of enzyme; A_t, A_{410} of the reaction product after 5 min of incubation with enzyme.

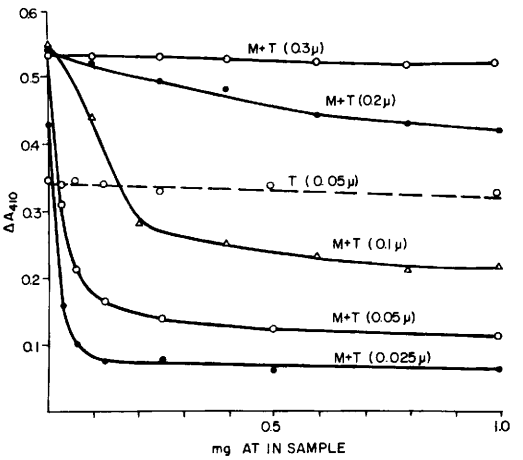

FIG. 3. Inactivation of M-catalase in various samples of crude extracts of *M. kansasii* TMC 1201 containing both M- and T-catalases after 2 h of preincubation with graded doses of AT. μ, Units of catalase employed in sample. Broken line represents response of pure T-catalase.

TABLE 2. Activity of T-catalase alone or in the presence of M-catalase in the routine assay after treatment with AT or precipitation with antiserum to the T-catalase of *M. kansasii* TMC 1201

Catalase class(es)	Treatment	*M. kansasii* TMC 1201			*M. scrofulaceum* TMC 1314		
		No. of replicates	$\Delta A_{410}/U^a$	Recovery (%)	No. of replicates	$\Delta A_{410}/U^a$	Recovery (%)
T only	Untreated	7	6.87 (±6.1)[b]	100	7	6.17 (±7.9)	100
	AT[c]	2	6.18 (±7.6)	90.0	3	4.94 (±8.8)	80.0
	Seroprecipitated[d]	5	5.14 (±11.5)	74.8	2	5.60 (±12.0)	90.8
M + T	AT[c]	6	2.03 (±7.3)		3	1.48 (±16.0)	
	Seroprecipitated[d]	5	1.59 (±13.4)		5	1.58 (±18.0)	

[a] Reduction in A_{410} in the H_2O_2 assay per unit of catalase in the reaction mixture.
[b] The numbers in parentheses are standard deviations, expressed as percentages of the means.
[c] Catalase in 100 μl was incubated with 1 mg of AT for 2 h before 50 μl of substrate was added.
[d] Expressed as the maximum activity observed in the precipitate after reaction of the catalase with serial dilutions of serum.

ΔA_{410} that was only one-third that of 1 U of isolated T-catalase.

The other approach to estimating T-catalase in the presence of M-catalase was based on serological titration of crude mixtures with the anti-T-catalase serum and measuring the catalase activity in the precipitate at the equivalence point. These experiments confirmed that T-catalase accounted for about one-third of the total catalase in the crude mixture from *M. kansasii* TMC 1201 (Table 2).

These experiments were extended to include a comparison of the efficiency and reproducibility of the two methods for estimating the T-catalase in crude extracts of both *M. kansasii* TMC 1201 and *M. scrofulaceum* TMC 1314 with the results obtained with T-catalase that had been separated chromatographically from the M-catalase. Both methods exhibited 75 to 90% efficiency in measurements of T-catalase in the absence of M-catalase, although the standard deviations (expressed as percentages of the mean activity values) were greater for the seroprecipitation method. There was striking agreement in measurements of the relative proportions of T-catalase in the crude mixtures from both strains tested by either method; i.e., the activity of 1 U of crude material (expressed as ΔA_{410}) corresponded to only 0.28 to 0.33 U after inactivation of M-catalase with AT or after the T-catalase was separated and assayed as an antigen-antibody precipitate (Table 2).

Selection of strategies for determining ImDs of catalases in crude mycobacterial extracts. The antiserum to the T-catalase of *M. kansasii* TMC 1201 was titrated by the simple supernatant and precipitate methods against chromatographically separated T-catalases from *M. kansasii* TMC 1201 and *M. scrofulaceum* TMC 1314, as well as by the supernatant-AT and precipitate methods against crude extracts of these organisms that contained both M- and T-catalases (Table 3). The binding capacities determined for the homologous enzyme were almost identical by all methods. The apparent binding capacities of the heterologous *M. scrofulaceum* catalase were 12 to 16% higher when the crude mixture was used than when the T-catalase alone was tested, but the homologous antigen showed little difference. This led to discrepancies of 17 and 23 U of ImD

TABLE 3. Homologous and heterologous titers of T-catalase alone and of T-catalase in the presence of M-catalase when antiserum raised against the T-catalase of *M. kansasii* TMC 1201 was used as a reference

Catalase class(es)	Titration technique	Titration of RS-114-29080 (log binding capacity) against:[a]		ImD[b]
		Homologous catalase (strain TMC 1201)	Heterologous catalase (strain TMC 1314)	
T only	Supernatant	2.01 ± 0.06[c]	1.37 ± 0.06	64
T only	Precipitate	1.83 ± 0.17	1.31 ± 0.05	52
T + M	Supernatant-AT[d]	2.01 ± 0.07	1.54 ± 0.01	47
T + M	Precipitate	2.00 ± 0.06	1.59 ± 0.05	41

[a] Log binding capacity is expressed as the logarithm of the reciprocal dilution of reference serum (R5-114-29080) required to bind a standard amount of T-catalase (13).
[b] ImD is expressed as 100 times the difference between the homologous and heterologous log binding capacities.
[c] Mean ± standard deviation.
[d] AT was used to inhibit M-catalase, as described in the text.

TABLE 4. ImDs of T- and M-catalases from slowly growing mycobacteria, as determined against *M. avium* TMC 716 and *M. kansasii* TMC 1201

Species	Strain	ImDs with the following reference systems:[a]		
		M. avium TMC 716 T-catalase	*M. kansasii* TMC 1201 T-catalase	*M. kansasii* TMC 1201 M-catalase
M. tuberculosis	W196	97 ± 5[b]	145 ± 9	ND[c]
M. avium	TMC 716	Homologous	15 ± 7	ND
M. intracellulare	TMC 1406	14 ± 6	24 ± 8	ND
M. szulgai	TMC 1328	22 ± 8	30 ± 3	54 ± 9
M. simiae	TMC 1226	26 ± 8	44 ± 9	101 ± 4
M. kansasii	TMC 1201	29 ± 3	Homologous	Homologous
M. kansasii	TMC 1204	35 ± 3	−5 ± 5	−6 ± 3
M. gastri	W417	48 ± 2	6 ± 8	ND
M. gordonae	TMC 1324	51 ± 13	99 ± 7	96 ± 9
M. scrofulaceum	TMC 1314	55 ± 5[b]	51 ± 7	75 ± 7
M. asiaticum	TMC 803	50 ± 4	27 ± 2	61 ± 3
M. xenopi	TMC 1482	86 ± 12	72 ± 7	ND
M. marinum	TMC 1218	78 ± 3	93 ± 6	ND
M. nonchromogenicum	TMC 1481	ND	ND	99 ± 3
M. triviale	TMC 1453	ND	ND	108 ± 12
M. terrae	TMC 1450	ND	ND	143 ± 15

[a] Mean ± standard deviation.
[b] Data from reference 15.
[c] ND, Enzyme not demonstrable.

by the supernatant-AT and precipitate methods, respectively. Intraspecies standard deviations in the ImDs of mycobacterial catalases determined by the simple supernatant method have ranged from ±0.7 to ±21.4 in prior studies (15). Therefore, the supernatant-AT and precipitate methods were considered to be reasonable alternatives to simple supernatant titration for extracts of the cells that contained both classes of enzyme; some caution was required in interpreting the absolute distances, but probably not the relative distances, between the two reference systems.

The following strategy was adopted for determining the ImDs of previously unstudied strains against selected reference antisera. New extracts were first assayed by the original first-order assay. Samples were then diluted appropriately, and 0.05 U was tested by the zero-order routine catalase assay. If the enzyme caused a ΔA_{410} of approximately 0.350, it was considered to be free of M-catalase, and the ImD was determined with the selected reference serum(a) by the simple supernatant titration. A ΔA_{410} markedly higher than 0.350 in the routine catalase assay was taken as evidence of a first-order reaction and indicated the presence of M- catalase. When this occurred, a sample containing 0.2 U of enzyme and 1 mg of AT in 0.1 ml of APB was incubated for 2 h, and then the zero-order assay was repeated. If the ΔA_{410} of this treated sample was greater than 0.250 U, there was sufficient T-catalase in the crude mixture to be titrated against appropriate antisera by the supernatant-AT method. The supernatant-AT method was also useful for titrating sera that exhibited intrinsic H_2O_2-destroying activity in high concentrations, which interfered with the assay for T-catalase.

If treatment of 0.2 U of crude enzyme with AT caused the ΔA_{410} to drop much below 0.250 U in the routine catalase assay, there was too low a proportion of T-catalase to be titrated by either of the methods described above. In that case, larger samples of crude extract were subjected to titration against reference antisera by the precipitate method to determine whether T-catalase could be detected at all, and if so, to establish its ImD for the reference systems. Titration of heat-treated extracts with anti-M-catalase serum is described above. Some T-catalase preparations that had not been examined in our prior studies were also titrated against the reference serum raised against *M. avium*, as reported previously (15).

The results of the serotitrations are shown in Table 4. The ImD of the T-catalase of *M. tuberculosis* to the T-catalase of *M. kansasii* when tested with the *M. kansasii* antiserum was 145 U. This is in very close agreement with the reciprocal value of 143 U reported in a previous study (14) in which *M. tuberculosis* antiserum was used. Similarly, the reciprocal values obtained between the reference sera to *M. tuberculosis* and *M. avium* were previously reported to be 98 and 97 ImD units, respectively (15). The reciprocal relationship between the *M. kansasii* and *M. avium* reference systems (15 ± 7 and 29

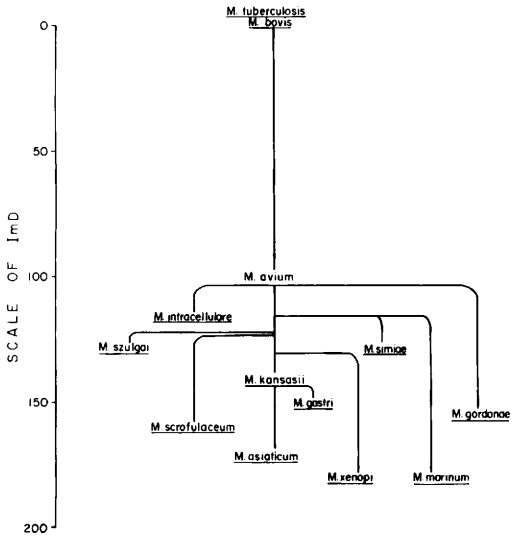

FIG. 4. Diagram of immunological relatedness of T-catalases of slowly growing mycobacteria. The vertical scale indicates the approximate ImD scores, whereas the horizontal expansion is only for convenience of display. The diagram is based on composite data from the present paper and two previous papers (14, 15).

± 3 U, respectively) is not as close as the other systems cited above. Nevertheless, their relative positions permit inferences to be made about the points of divergence of the T-catalases of the species tested. For example, the T-catalase of *M. scrofulaceum* is equidistant from the T-catalases of *M. avium* and *M. kansasii*, but each of the ImDs is greater than the distance between these two species; *M. scrofulaceum* is also markedly farther from *M. tuberculosis* than *M. kansasii* is. These data suggest that the point of divergence of *M. scrofulaceum* occurred between *M. avium* and *M. kansasii* and extended a considerable distance beyond both of these species. Data from this study, as well as mean species ImD scores from our prior two studies (14, 15), were used to construct a diagram of divergence of the catalases from the species examined to date (Fig. 4).

Of the species studied, only *M. terrae*, *M. triviale*, and *M. nonchromogenicum* failed to exhibit T-catalase, although all three of these species exhibited M-catalase. Conversely, *M. tuberculosis*, *M. bovis*, *M. intracellulare*, *M. avium*, *M. gastri*, *M. marinum*, and *M. xenopi* yielded T-catalase but no M-catalase. The remaining species produced both classes. Among five strains that exhibited both catalase classes, there was only a moderate correlation (correlation coefficient, 0.82) between the ImD scores against the T- and M-catalase reference systems of *M. kansasii*. In general, the M-catalases showed markedly greater ImD scores than the T-catalases, but even this relationship was not absolute.

With only one exception, all strains that produced both M- and T-catalases contained a sufficient proportion of the T-catalase to permit serological study by the supernatant-AT method. The extract of *M. gordonae* TMC 1324 exhibited a ratio of M-catalase to T-catalase of 7:1, so it was necessary to use the precipitate assay method to establish its ImD against the reference systems.

DISCUSSION

The kinetic studies described above have added another parameter to the criteria for distinguishing between the two classes of mycobacterial catalase. The M-catalase class, which has K_m values in the range from 143 to 156 mM H_2O_2, behaves much like the corresponding enzyme from mammalian tissues. The ubiquity of the M-catalases in nature is compatible with conservation of this enzyme during the evolution of eucaryotic aerobic forms of life (8). The presence of this enzyme in many of the highly aerobic mycobacteria is consistent with such conservation; however, the high ImD values observed with the M-catalase reference system suggest a high degree of structural divergence of this enzyme within the single genus *Mycobacterium*. It is possible that only a region of the molecule in close proximity to the functional group is highly conserved. Since we have demonstrated catalase function in seroprecipitates, it is evident that the antibody is not directed against the functional group itself.

The presence of a second class of catalases (T-catalases) with unusually low K_m values, either together with the more commonly recognized M-catalase class or alone as the sole H_2O_2-destroying component, does not appear to be unique to the genus *Mycobacterium*. Claiborne and Fridovich have reported that one of the two catalase classes which they isolated from *Escherichia coli* has a K_m of 3.9 mM H_2O_2 (4). This catalase, which they designated HP-I, also exhibits peroxidase activity at a neutral pH with *o*-dianisidine, guaiacol, *p*-phenylenediamine, pyrogallol, and catechol; the purified T-catalase from *M. tuberculosis* acts as a peroxidase at pH 7.5 on *o*-dianisidine, pyrogallol, and catechol but not on guaiacol (6, 7).

Differences on tolerance to heat and to the enzyme inhibitor AT and the retention of activity of seroprecipitates have made it possible to adapt specific serological assay techniques to the study of one class of catalase in the presence of the other. This elimination of the need to subject the crude cell extracts to complex frac-

tionation procedures has facilitated the expansion of the serological studies to a broad range of species in the genus *Mycobacterium*. Although more than two bands of catalase activity have been described in electrophoretograms of extracts of some mycobacterial strains (14), it is probable that some of these bands reflect different states of aggregation of the same enzyme. This interpretation is supported by the detection of two peaks of M-catalase during diethylaminoethyl-Sephadex fractionation of an *M. kansasii* extract (Fig. 1), which were indistinguishable from one another serologically.

The addition of a second T-catalase reference system (15) permitted estimation of direction of divergence to be added to the simple estimation of degree of divergence that was possible in our earlier study with a single reference system (14). The addition of a third system now permits estimation of the points at which branching of the train of divergence of this enzyme occurs. It is not appropriate to assume that the evolutionary divergence of the T-catalase molecule is a direct and accurate reflection of the evolution of the species within the genus. As shown by Schleifer et al. (12), the rate of evolution of the gene coding for catalase in the genus *Staphylococcus* does not coincide with the rate of evolution of the total genome, as reflected by deoxyribonucleic acid homology measurements. On the other hand, Norgard and Imaeda (10) have identified fractions of deoxyribonucleic acids from strains of *M. smegmatis* with different degrees of homology to one another, so even homology data must be interpreted cautiously. Each system of analysis adds a tile to the evolutionary mosaic.

Nevertheless, examination of the T-catalase divergence diagram (Fig. 4) does suggest some reflection of an evolutionary direction within the genus. At one extreme of the scale are *M. tuberculosis* and *M. bovis*, which are not found free-living in nature but are found essentially in warm-blooded hosts. Centrally on the diagram are *M. avium*, *M. intracellulare*, and *M. kansasii*, which occur not only free in nature but also frequently as the causes of serious diseases in birds and mammals (17). *M. scrofulaceum* is also found free in nature but is less frequently associated with disease, which is usually a fairly self-limiting localized cervical adenitis in children (17). *M. simiae* and *M. szulgai* have also rarely caused human disease, but the distribution of these species in nature is not yet well documented. *M. gastri* is not a pathogen. Farther down the scale of T-catalase divergence are *M. marinum* and *M. xenopi*, both of which are found predominantly in water. These species are opportunistic pathogens; *M. marinum* causes self-limiting, fairly benign skin lesions in individuals who incur abrasions in contaminated waters (17). *M. asiaticum* has been isolated too infrequently to permit assessment of its normal habitat and pathogenic potential. Also near the bottom of the scale is *M. gordonae*, a waterborne organism not considered to be a pathogen (17). Three other species, *M. terrae*, *M. nonchromogenicum*, and *M. triviale*, are also rarely, if ever, associated with disease, and we were unable to demonstrate any T-catalase in extracts of these organisms. However, the M-catalases of these species exhibited ImD scores comparable to that of *M. gordonae* when tested against the *M. kansasii* M-catalase reference system. In summary, there does appear to be some correlation between the position of species on the diagram of divergence of their T-catalases and the evolution of these species from free-living, nonpathogenic organisms toward increasingly obligatory pathogenicity.

The estimation of degree of concordance between serological distance relationships of T-catalases and M-catalases will require the development of additional M-catalase reference systems. Development of these systems should also provide some further insights into the evolutionary positions of the few mycobacterial species that do not produce T-catalase.

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