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Radiometric Studies with Gas-Liquid and Thin-Layer Chromatography for Rapid Demonstration of Hemin Dependence and Characterization of *Mycobacterium haemophilum*

JAMES J. DAMATO[1]†* AND MICHAEL T. COLLINS[2]

Department of Microbiology and Environmental Health, Colorado State University, Fort Collins, Colorado 80523,[1] and Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin—Madison, Madison, Wisconsin 53706[2]

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Eight isolates of *Mycobacterium haemophilum* were evaluated by radiometric methods to determine whether this test system could support the growth of these organisms as well as demonstrate their growth requirements for iron complexes such as hemin, ferric ammonium citrate, and blood. In addition, gas-liquid and thin-layer chromatography were evaluated to determine whether these procedures could further differentiate *M. haemophilum* from other mycobacteria. During the initial 24 to 48 h, there was no significant difference between the radiometric test broths containing iron complexes and control broths without iron supplementation. After 48 h, the test growth index readings rapidly increased, and control broth readings leveled off and declined. The mean growth index reading of the test broths after 6 days of incubation was 100 times that of the controls. The mean incubation time with supplemented 7H10 agar was 17 days. The use of radiometric media resulted in the demonstration of hemin dependence by *M. haemophilum* significantly earlier than with 7H10 agar. Of the three supplements studied, whole blood provided the greatest growth rate, followed by ferric ammonium citrate and hemin. When 12 species of mycobacteria other than *M. haemophilum* were radiometrically evaluated, no isolate demonstrated an iron complex requirement. Gas-liquid and thin-layer chromatography procedures were able to rapidly differentiate *M. haemophilum* from the other 12 *Mycobacterium* species.

Mycobacterium haemophilum is unique among mycobacteria because of its hemin growth requirement. Sompolinsky et al. described this species and demonstrated that it was pathogenic for humans (9, 10). Subsequent studies substantiated these preliminary observations (2, 3, 5, 7, 11). Previous studies employed a variety of media supplemented with hemin or other iron-containing compounds; however, with these media, 2 to 4 weeks of incubation were required before *M. haemophilum* was detected (2, 7, 8). Since BACTEC radiometric methodology has demonstrated the ability to rapidly detect mycobacteria (1, 4, 6), these procedures were evaluated to determine whether a radiometric system could support the growth of *M. haemophilum* and demonstrate the growth requirement of the organism for hemin or iron-containing compounds. In addition, gas-liquid chromatography (GLC) and thin-layer (TLC) chromatography were evaluated to determine whether these procedures could further differentiate *M. haemophilum* from other mycobacteria.

MATERIALS AND METHODS

Sixty-eight mycobacterial isolates were used in this study. Of the eight *M. haemophilum* isolates evaluated, seven were provided by David J. Dawson (Laboratory of Microbiology and Pathology, State Health Laboratory, Brisbane Q, Australia). An additional isolate was obtained from the Trudeau Mycobacterial Stock Culture Collection, TMC 804 (National Jewish Hospital and Research Center, Denver, Colo.) All remaining mycobacteria were provided by the Fitzsimons Army Medical Center, Aurora, Colo. They included five isolates each of *M. tuberculosis*, *M. bovis*, *M. kansasii*, *M. scrofulaceum*, *M. szulgai*, *M. flavescens*, *M. gordonae*, *M. avium/intracellulare* complex, *M. gastri*, *M. fortuitum*, *M. chelonei*, and *M. pheli*.

Each *M. haemophilum* isolate was initially cultured on Middlebrook 7H10 agar (7H10) (BBL Microbiology Systems, Cockeysville, Md.) containing 60 μM hemin, and a 7H10 agar control plate without hemin, to provide an actively growing isolate and to confirm its hemin growth requirement. The other mycobacteria were subcultured on 7H10 agar. All 68 organisms were subsequently suspended in a solution containing 0.2% fatty acid-free albumin and 0.02% polysorbate 80, and the suspension density was adjusted to equal the 0.5 McFarland turbidity standard. A 0.1-ml sample was then inoculated into BACTEC Middlebrook 7H12 radiometric test broth (7H12) (Johnston Laboratories, Cockeysville, Md.) containing 60 μM hemin, 1.5% ferric ammonium citrate (FAC), or 5% (vol/vol) fresh hemolyzed human blood without anticoagulants. A 7H10 agar plate containing 60 μM hemin was also inoculated at this time. The same media without iron supplements were inoculated as negative controls. All media were incubated at 32°C. The BACTEC broths were read daily for 12 days with a BACTEC model 301, and the growth index readings were recorded. A growth index (GI) reading of 100 was equivalent to 0.025 μCi of liberated $^{14}CO_2$. Plate media were placed in polyethylene bags (4 by 6 in. [10.2 by 15.2 cm]) and examined weekly for 4 weeks. All *M. haemophilum* cultures were confirmed by a positive fluorescent auramine stain (Difco Laboratories, Detroit, Mich.), by demonstration of a hemin growth requirement, and by no growth when subcultured on tryptic soy agar (Difco).

GLC and TLC studies were performed as described by

* Corresponding author.
† Present address: Department of Pathology, Fitzsimons Army Medical Center, Aurora, CO 80045.

Knisley (M.S. thesis, Colorado State University, Fort Collins, 1982). Approximately 10 mg of whole mycobacterial cells was washed twice with 5 ml of double-distilled water. After the final wash, the organisms were resuspended in 2 ml of double-distilled water, autoclaved for 15 min at 121°C, and dried. The dried cells were then methanolated by the addition of 2 ml of methanol–toluene–concentrated H_2SO_4(30:15:1). This suspension was sonicated for 20 min and incubated at 75°C for 18 to 24 h. The test products were extracted twice with 2 ml of redistilled hexane, and the extracts were combined. Approximately 100 to 200 mg of ammonium bicarbonate was added until the extract was adjusted to pH 7.

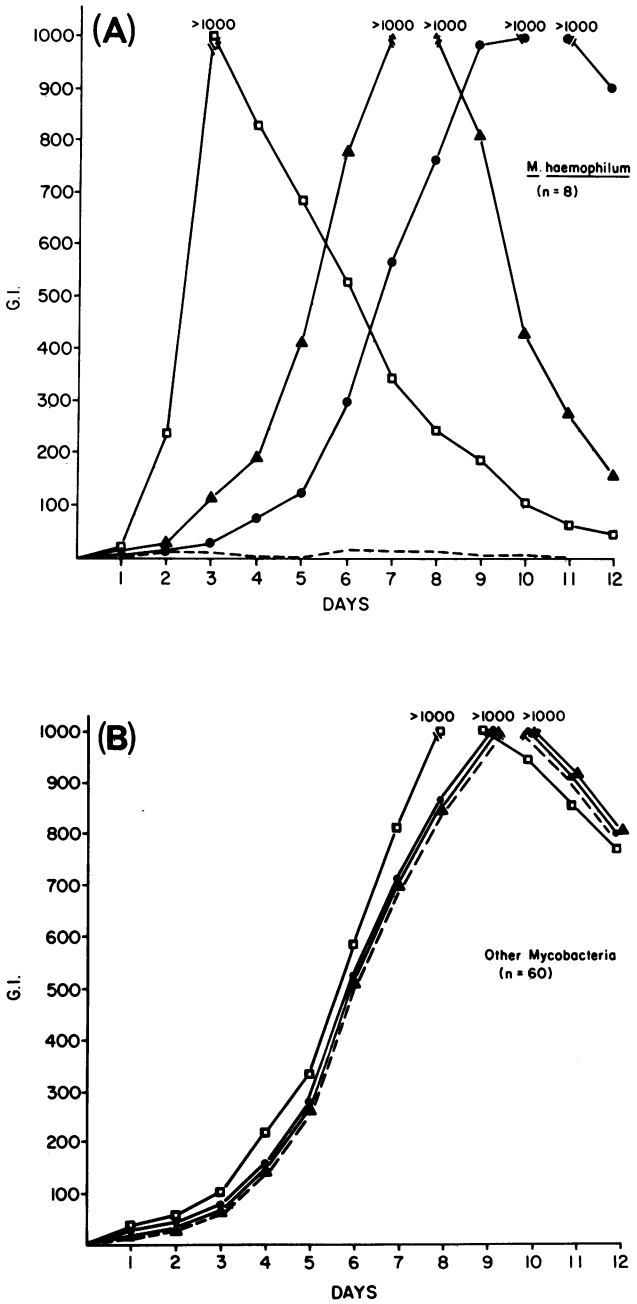

FIG. 1. Effects of various iron supplements on the growth of *M. haemophilum* (A) and other mycobacteria (B). Symbols indicate radiometric broths without iron complex supplements (– – –) and radiometric broths supplemented with whole human blood (5% [vol/vol]) (□), 1.5% ferric ammonium citrate (▲), and 60 μM hemin (●).

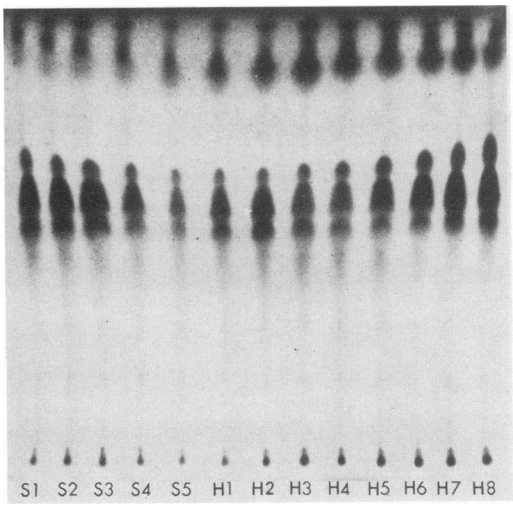

FIG. 2. TLC results for *M. szulgai* (S1 to S5) and *M. haemophilum* (H1 to H8).

A Pasteur pipette lightly packed with glass wool was used to separate the ammonium bicarbonate, and the filtrate was evaporated to dryness with a stream of nitrogen. The dried extract was suspended in approximately 0.5 ml of double-distilled hexane. GLC studies of the fatty acid methyl esters were performed with a Varian Aerograph 3700 and a Varian Recorder 9176. Approximately 1 μl of the hexane test solution was injected into a 1.8 stainless steel column (inside diameter, 2 mm) packed with 3% OV-1 on 80/100 Supelcoport (Supelco, Inc., Bellefonte, Pa.).

A second reference column was used to adjust the base line to zero and to compensate for temperature drift. Injector and detector port temperatures for both columns were 330 and 350°C, respectively. The temperature program was 190°C for 5 min, increasing at 4°C/min to a final temperature of 310°C for 10 min, and the flow rates were 300 ml of air, 30 ml of H_2, and 45 ml of N_2 carrier gas per ml. The detector sensitivity was set at 256×10^{-11}.

TLC of the methyl mycolates was performed with silica gel G and $CaSO_4$ binder (Analtech, Inc., Newark, Del.). The plates were spotted with ca. 50 μl of methyl ester preparation, and the solvent system was hexane-diethyl ether (78:22). Plates were air dried and sprayed with 0.6% $K(CrO_3O_2)$ in 55% H_2SO_4 and heated at 150°C in an oven for 15 to 20 min.

RESULTS

The effects of the various iron supplements on the eight *M. haemophilum* isolates are shown in Fig. 1a. After approximately 24 to 48 h, the radiometric broths containing iron compounds demonstrated a rapid GI increase, whereas that of the control broths lacking iron supplementation remained low or declined. The *M. haemophilum* cultured in the radiometric broth with hemolyzed human blood demonstrated the highest metabolic activity, followed by FAC and hemin. Regardless of the method of iron supplementation, after 4 days of incubation, the test broths had GI readings 20 or more times greater than those of the non-iron-supplemented control broths.

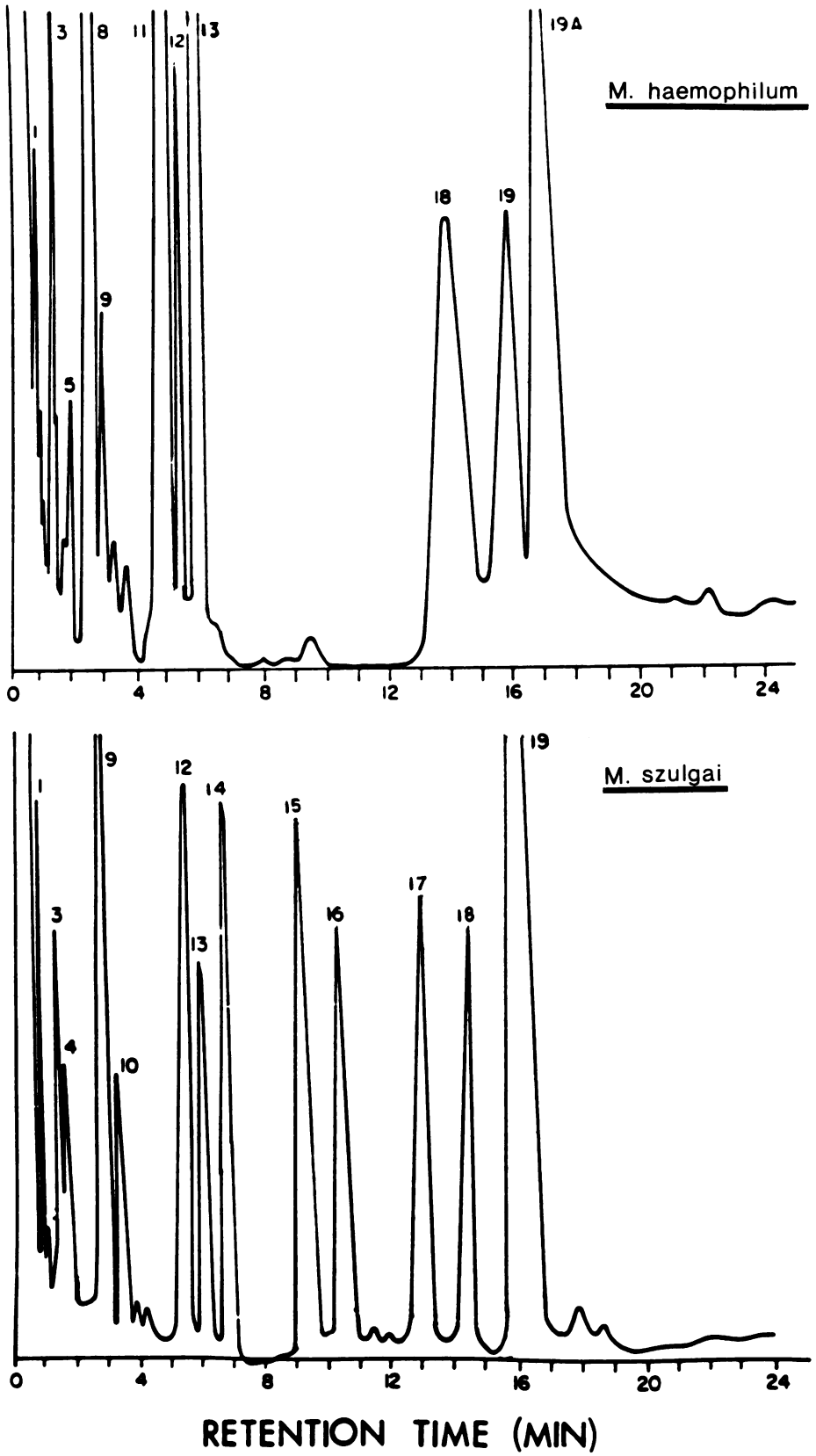

FIG. 3. Representative GLC results for *M. haemophilum* (n = 8) and *M. szulgai* (n = 5) isolates.

The effects of the various iron supplements on the growth of mycobacteria other than *M. haemophilum* are shown in Fig. 1b. Although some species differences in GI readings were observed in the test broth containing human blood, the overall GI readings for the control and test broths paralleled each other throughout the test period.

TLC patterns for *M. haemophilum* isolates were sufficiently unique to permit differentiation of this organism from most other mycobacteria tested. The one exception occurred with the isolates of *M. szulgai* (Fig. 2).

GLC results, however, permitted differentiation of these two species. Representative GLC fatty acid and methyl ester analyses of *M. haemophilum* and *M. szulgai* are shown in Fig. 3. *M. haemophilum* had peaks 5, 8, 11, and 19A, but *M. szulgai* lacked these peaks. Conversely, *M. szulgai* had peaks 4, 10, 14, 15, 16, and 17, which were not observed with the *M. haemophilum* isolates. In addition, none of the other mycobacteria tested demonstrated the peak triplet 15, 16, and 17.

DISCUSSION

The BACTEC radiometric test system was able to support the growth of all eight *M. haemophilum* isolates and rapidly demonstrated the unique iron growth requirements of the organism. Regardless of the iron supplement used in the study, after 4 days of incubation all test broths demonstrated increasing GI readings, whereas those of control broths leveled off and began to decline. The mean detection time for *M. haemophilum* growth in a conventional medium (7H10 agar plus hemin) was 17 days. Each of the three supplements used with the BACTEC test system permitted rapid growth of the isolates (2 to 5 days), with 5% hemolyzed whole human blood stimulating the most rapid growth, followed by FAC and hemin. Although there was no iron growth dependence demonstrated by the 60 isolates of mycobacteria other than *M. haemophilum* (Fig. 1b), there appeared to be somewhat faster growth in the radiometric broths supplemented with human blood. Differences observed with various supplements could be attributed to facilitated iron uptake by some factor(s) associated with the whole blood, or this supplement may have provided additional nutrients which permitted an enhanced growth rate. Previous studies involving solid media also demonstrated varied growth rates, depending on the particular supplement and medium selected (3, 9). Dawson et al. reported that when 5% sheep blood agar or Lowenstein Jensen medium (1.5% FAC) was used, growth of *M. haemophilum* was observed earlier than with Middlebrook 7H9 (1.5% FAC), Middlebrook 7H10 (60 μM hemin), or chocolate agar (3). Low levels of metabolic activity of *M. haemophilum* were detected in control broths without iron supplementation. This could have been due to carry-over of minimal amounts of iron with the initial test inoculum, or to the fact that commercial BACTEC broths contain 40 μg of FAC per ml. Although the concentration of this medium component was not sufficient to sustain significant growth, it may have been adequate to permit limited metabolic activity. Serial dilutions of the inoculum with diluents lacking iron complexes could be a method of minimizing iron complex carry-over, thereby further enhancing the difference in GI readings.

It was critical that the *M. haemophilum* inoculum be prepared from an actively growing culture adjusted to 0.5 McFarland standard. If the radiometric broths received too heavy an inoculum, the growth curves shown in Fig. 1 were obscured by high initial control broth readings. Properly standardized inocula provided GI readings of ca. 15 or less after 24 h of incubation. If initial radiometric readings were high, the sample was diluted and the test procedure was repeated.

Using the GLC and TLC procedures described by Knisley et al., it was possible to rapidly differentiate the *M. haemophilum* from the vast majority of other mycobacteria studied within 48 h after isolation. Although successful GLC and TLC tests were performed on organisms harvested from plate cultures, preliminary studies have shown that such testing may also be feasible with 1 to 5 mg of cells grown in Middlebrook 7H10 Tween broth supplemented with 60 μM hemin.

This study was limited by the availability of *M. haemophilum* isolates, and only 12 other mycobacteria species were examined; however, it illustrated the potential that radiometric, GLC, and TLC methodologies have for the rapid detection and identification of *M. haemophilum*. Hopefully, these procedures will contribute to the enhanced detection of this opportunistic pathogen in clinical specimens.

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