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Microaerophilic Conditions Promote Growth of *Mycobacterium genavense*

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Our studies show that microaerophilic conditions promote the growth of *Mycobacterium genavense* in semisolid medium. The growth of *M. genavense* at 2.5 or 5% oxygen was superior to that obtained at 21% oxygen in BACTEC primary cultures (Middlebrook 7H12, pH 6.0, without additives). By using nondecontaminated specimens, it was possible to detect growth with very small inocula (25 bacilli/ml) of 12 different *M. genavense* strains (from nude mice) within 6 weeks of incubation under low oxygen tension; conversely, with 21% oxygen, no growth of 8 of 12 (66.7%) *M. genavense* strains was detected (growth index, <10). The same beneficial effect of 2.5 or 5% oxygen was observed in primary cultures of a decontaminated clinical specimen. Low oxygen tension (2.5 or 5%) is recommended for the primary isolation of *M. genavense*. Microaerophilic cultivation of other atypical mycobacteria, especially slow-growing (e.g., *Mycobacterium avium*) and difficult-to-grow (e.g., *Mycobacterium ulcerans*) species, is discussed.

Mycobacteria are generally considered obligate aerobes (25) that grow at oxygen tensions ranging from atmospheric to microaerophilic conditions (17, 24). Routinely, primary cultures are incubated in room air or air containing 10% CO_2. Increased CO_2 tension produces earlier and more luxuriant growth of *Mycobacterium tuberculosis* (18), especially on agar media. In the BACTEC system, the recommended atmosphere for mycobacteria is 5 to 10% CO_2 in air (39). Primary cultures of all mycobacteria, including strict pathogens (e.g., *M. tuberculosis*), saprophytic mycobacteria other than *M. tuberculosis* (e.g., *Mycobacterium gordonae*), opportunistic mycobacteria other than *M. tuberculosis* (e.g., *Mycobacterium avium*), and even difficult-to-grow mycobacteria (e.g., *Mycobacterium genavense* and *Mycobacterium ulcerans*), are incubated under the same conditions (room air or 5 to 10% CO_2 in air). *M. genavense* is a fastidious mycobacterium which most commonly causes disease in AIDS patients (3, 5, 7, 14, 27), but there are recent reports of *M. genavense* infections in patients without human immunodeficiency virus (2, 22). *M. genavense* also infects birds (15, 16, 31, 32) and dogs (19). Approximately 50% of *M. genavense* isolates grow in liquid media such as Middlebrook 7H12 (5). By radiometry, Hoop et al. (16) cultured *M. genavense* from 67.6% of bird tissues (livers and intestines positive for acid-fast bacilli [AFB] by microscopy). Results from radiometric assays can require 2 months or more (7, 16). Growth may be improved at pH 6.0 (4, 15, 16, 40, 41). We recently demonstrated that culture conditions recommended for *M. tuberculosis* (polyoxyethylene stearate and PANTA [polymyxin B-amphotericin B-nalidixic acid-trimethoprim-azlocillin] at pH 6.8) inhibit primary isolation of *M. genavense* in Middlebrook 7H12, and we recommended the use of medium at pH 6.0 without additives in the BACTEC system (34). Solid media such as Löwenstein-Jensen and Middlebrook agar are not suitable for the isolation of *M. genavense* (16). Designation of mycobacteria as fastidious may reflect only, for example, that the culture medium or pH is inadequate, as demonstrated for *M. genavense*, but in addition may involve inappropriate oxygen tensions.

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Surprisingly, microaerophilic conditions have seldom been applied to the primary isolation of mycobacteria. Franzblau (10) incubated *Mycobacterium leprae* in liquid culture at an oxygen concentration of 2.5% instead of 21% to maintain its metabolic activity in the BACTEC system. Because it is likely that *M. genavense* is disseminated from the gut (9), an excellent site for anaerobic and microaerophilic bacteria, we attempted to improve the growth of primary cultures of *M. genavense* by modifying oxygen tensions. We report here the behavior of *M. genavense* in a semisolid medium and the influence of different oxygen concentrations on its growth in the BACTEC system.

MATERIALS AND METHODS

***M. genavense* strains.** Twelve strains of *M. genavense* were studied. ITM (Institute of Tropical Medicine) 95-975, 96-1283, 96-1438, 96-1439, and 96-1799 were isolated from five different AIDS patients living in Geneva, Switzerland; ITM 96-823 and 97-76 were isolated from AIDS patients from Iowa City, Iowa; and ITM 97-75 was isolated from an AIDS patient from Seattle, Wash. Four other strains originated from birds living in the Antwerp Zoo, Antwerp, Belgium: ITM 95-610, from a cutthroat weaver (*Amadina fasciata*); ITM 95-614, from a gouldian finch (*Chloebia gouldiae*); ITM 95-615, from a zebra finch (*Poephila guttata castanotis*); and ITM 96-6, from a turquoise tanager (*Tangara mexicana*). The organisms were recovered from lungs and spleens of nude mice (BALB/c; IFFA Credo, Lyon, France) inoculated intraperitoneally 8 to 9 months previously, as previously described (34). The AFB were counted by the method of Shepard and McRae (38). Dilutions of tissue homogenates were made in phosphate-buffered saline (PBS). No decontamination was performed.

Clinical specimen. Liver (ITM BK97-1056) (kept at −20°C) from an African silver-bill (*Euodice cantans*) kept in the Antwerp Zoo was sent to us because the contributor was unable to obtain growth on Ogawa and Löwenstein-Jensen media even though the tissues contained large numbers of AFB. These AFB were identified as *M. genavense* by characterization of specific sequences in the *rrn* operon (data not shown). The specimen was thawed, minced, and homogenized with a pestle and mortar in PBS. The tissue homogenate was centrifuged differentially to remove the larger tissue debris (100 × *g*, 5 min, 10°C). The supernatant was then centrifuged at 2,700 × *g* for 45 min at 10°C, and the resulting pellet containing the AFB was resuspended in PBS. AFB were counted as described above. The bacterial suspension was divided into four aliquots, each decontaminated by one of the following procedures: (i) sodium dodecyl sulfate (SDS) (35), (ii) *N*-acetyl-L-cysteine-sodium hydroxide (NALC) (25), (iii) 4% sodium hydroxide (Petroff [28]), or (iv) 1 N hydrochloric acid (HCl) followed by neutralization with 4% sodium hydroxide.

Culture media and gas conditions. Semisolid medium (23) was used after adjusting the pH to 6.0 with phosphoric acid. This medium was inoculated with 0.2 ml of a suspension of 10^6 AFB/ml, inverted repeatedly to avoid formation of bubbles and aeration, and incubated at 37°C in air for 2 months.

BACTEC pyrazinamide control medium (PZA) (pH 6.0 ± 0.2), without polyoxyethylene stearate or PANTA (34), was inoculated in triplicate with 100 μl of a nondecontaminated *M. genavense* suspension from nude mouse organs to

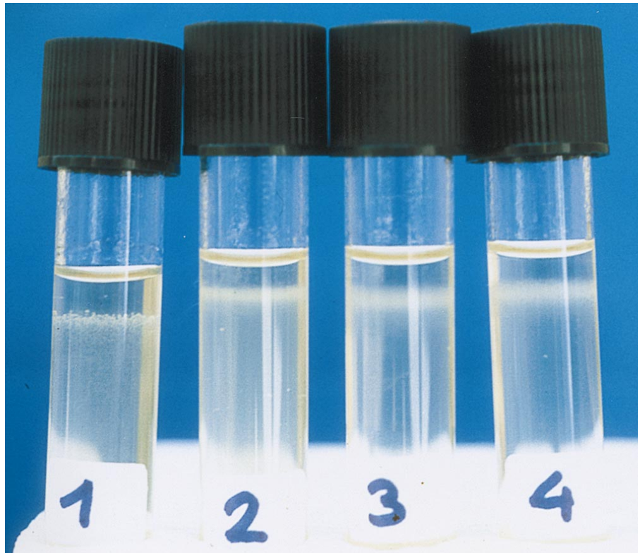

FIG. 1. Microaerophilic nature of *M. genavense* primary cultures in Marks semisolid medium. Vials: 1, ITM 97-75; 2, ITM 95-975; 3, ITM 96-6; 4, ITM 95-610.

obtain 10^2 to 10^5 AFB/vial and incubated at 37°C. Each aliquot of the clinical specimen decontaminated by one of the above methods was inoculated into a single vial for each of the experimental conditions tested. Readings of the release of $^{14}CO_2$ in the BACTEC 460 TB instrument were made twice a week for 6 weeks or until the growth index (GI) reached 999. Three different gas mixtures (Praxair NV, Oevel, Belgium) were used: (i) 10% CO_2 in air (21% O_2), (ii) 5% O_2–10% CO_2–85% N_2, and (iii) 2.5% O_2–10% CO_2–87.5% N_2. When the GI reached 999, 0.2 ml of the vial was inoculated into thioglycollate medium, blood agar, and Middlebrook 7H11. Ziehl-Neelsen staining was performed on the BACTEC vials when the GI reached 999 and on the semisolid medium.

RESULTS

Growth in semisolid medium. All *M. genavense* strains were microaerophilic in semisolid medium: growth appeared as a 3- to 5-mm zone, with the superior limit remaining 3 to 7 mm under the surface of the culture medium. Figure 1 shows the microaerophilic natures of four different strains of *M. genavense.*

TABLE 1. Influence of oxygen concentration on growth of 12 strains of *M. genavense* (from nude mice) in the BACTEC system[a]

Inoculum (no. of AFB/vial)	No. of strains reaching GI of 999 within 6 wk of incubation under oxygen concn:		
	21%	5%	2.5%
10^5	11	12	12
10^4	8	12	12
10^3	4	12	12
10^2	2	12	12

[a] Middlebrook 7H12, pH 6.0, without additives.

Growth in the BACTEC system. Table 1 shows the number of strains reaching a GI of 999 in the BACTEC system within 6 weeks of incubation under the three different oxygen concentrations: 2.5, 5, and 21%. The 12 strains reached a GI of 999 within 6 weeks (the time routinely required to retain the vials [39]) under low oxygen concentrations, even with small inocula (10^2 AFB/vial). By comparison, when air is used to gas the vials, growth of *M. genavense* is inhibited. This is most obvious with smaller inocula: for example, at 10^2 AFB/vial, only 2 of 12 (16.7%) strains reached a GI of 999 within 6 weeks.

An illustrative example from Table 1 is shown in Fig. 2, demonstrating bacterial growth with inocula of 10^5, 10^4, 10^3, and 10^2 AFB/vial of strain ITM 96-1283 in the BACTEC system in the presence of 21, 5, or 2.5% oxygen in the gas mixture. The impact of lower oxygen tension on growth can be better appreciated with smaller inocula. Growth of *M. genavense* ITM 96-1283 was detected earlier with 5 or 2.5% oxygen than with 21% oxygen; for example, with an inoculum of 10^4 AFB/vial, the GI reached 999 in 13 days under 2.5 or 5% O_2 versus 23 days with 21% O_2. Moreover, even with smaller inocula, of 10^2 and 10^3 AFB/vial, a difference was observed between 2.5 and 5% O_2. With an inoculum of 10^2 AFB/vial, the growth of *M. genavense* ITM 96-1283, based on the time needed to reach a GI of 999, was higher in the presence of 2.5% O_2 (23 days) than 5% O_2 (34 days).

Figure 3 shows the growth of the 12 strains with the three

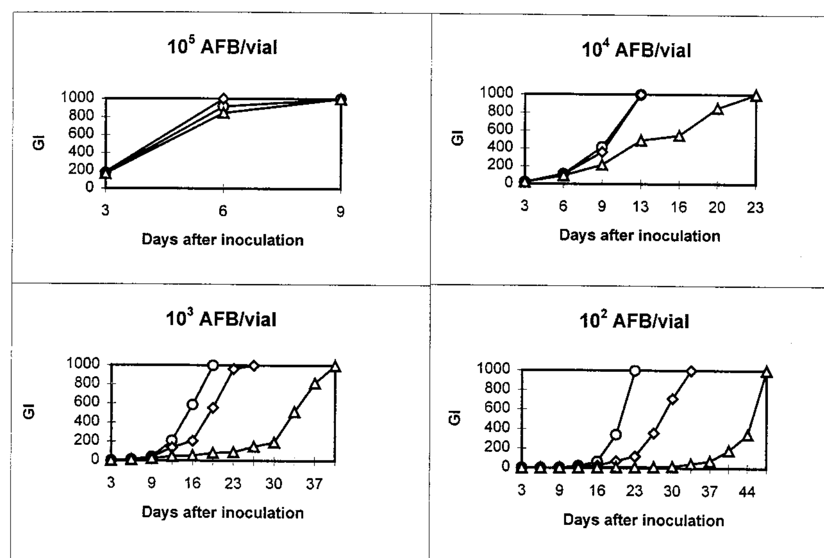

FIG. 2. Effects of oxygen on growth of different inocula of *M. genavense* ITM 96-1283 in the BACTEC system (Middlebrook 7H12, pH 6.0, without additives). ○, 2.5% O_2; ◇, 5% O_2; △, 21% O_2.

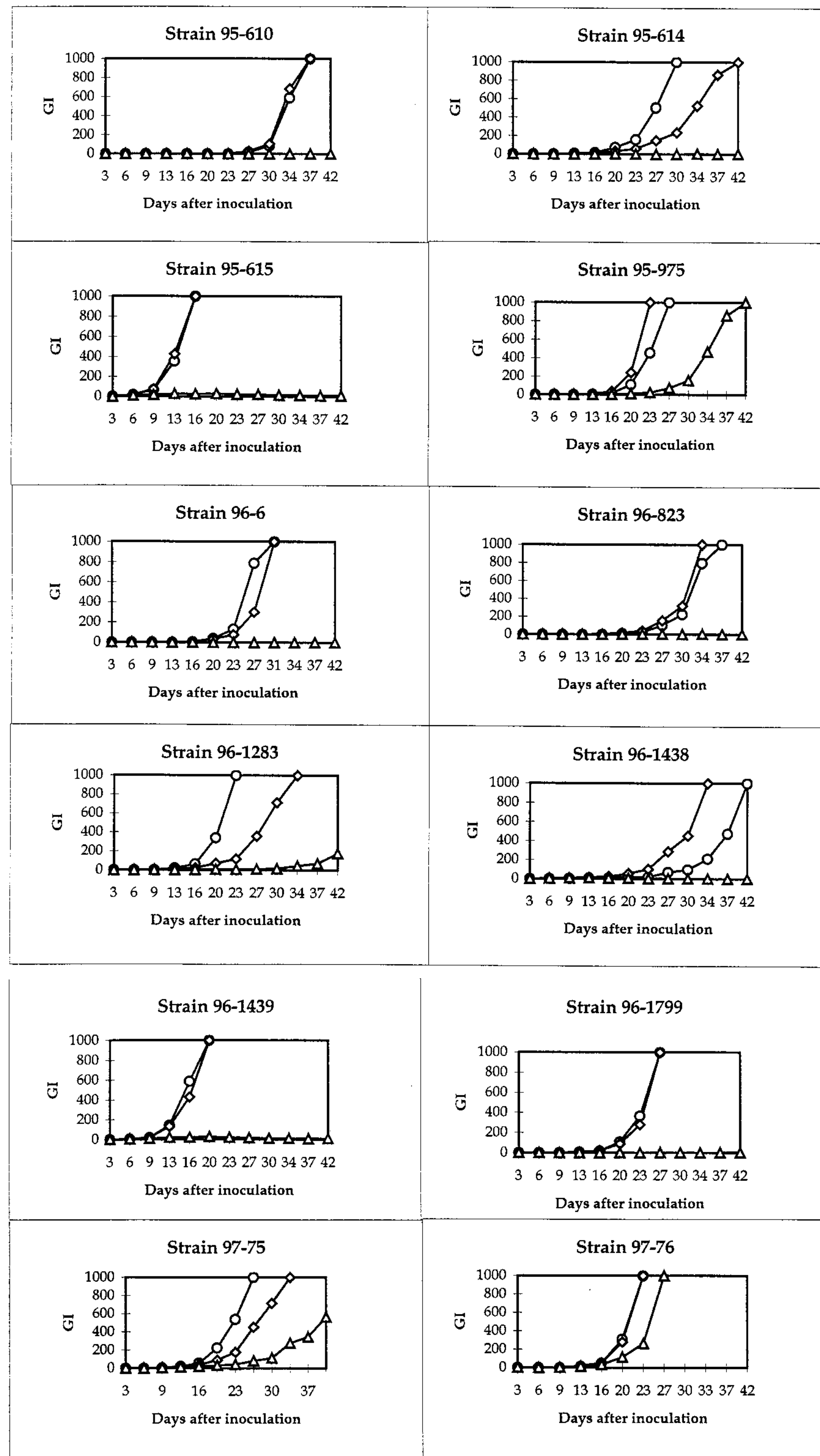

FIG. 3. Effects of different oxygen concentrations on growth of 12 *M. genavense* strains (10^2 AFB in PZA) in BACTEC primary cultures within 6 weeks of incubation. ○, 2.5% O_2; ◇, 5% O_2; △, 21% O_2.

different gas mixtures following inoculation of 10^2 AFB/vial in the BACTEC system. For all strains tested, earlier growth was detected under 5 or 2.5% O_2 than under 21% O_2. For 8 of the 12 strains, there was no growth after 6 weeks of incubation (42 days) in the presence of 21% O_2. There are some differences between the strains: strains ITM 95-975, 96-823, and 96-1438 grew better at 5% O_2 and strains ITM 95-614, 96-1283, and 97-75 had higher growth rates in the BACTEC system with

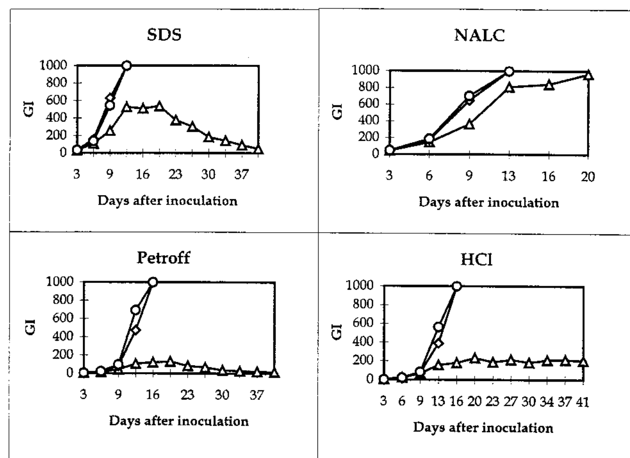

FIG. 4. Influence of oxygen concentration on primary cultures of an *M. genavense* clinical specimen (10^6 AFB in PZA) decontaminated by SDS, NALC, the Petroff method, or HCl in the BACTEC system. ○, 2.5% O_2; ◇, 5% O_2; △, 21% O_2.

2.5% O_2, while the growth curves for strains ITM 95-610, 95-615, 96-6, 96-1439, 96-1799, and 97-76 at 5 and 2.5% O_2 were similar. Multiplication of all *M. genavense* strains was detected in the BACTEC system in less than 6 weeks under the lower oxygen concentrations.

The positive impact of low oxygen concentrations on *M. genavense* growth in the BACTEC system was observed with the clinical specimen. Figure 4 shows growth curves obtained with the three different gas mixtures when the vials were inoculated with a specimen (10^6 AFB/vial) decontaminated by SDS, NALC, the Petroff method, or HCl. Better growth was obtained with 2.5 or 5% O_2 than with 21% O_2 for each of the four decontamination methods. The GI reached 999 with all methods at low oxygen concentrations but never reached 999 at 21% oxygen when SDS, the Petroff method, or HCl were applied.

In Fig. 5, the four decontamination methods were compared at 2.5% oxygen. Earlier detection of growth was observed when the specimen was decontaminated with NALC or SDS than by the Petroff method or HCl.

Thioglycollate, blood agar, and Middlebrook 7H11 subcultures from BACTEC vials showed all vials to be free of contaminating organisms.

DISCUSSION

Most aerobic bacteria may in fact be microaerophilic, requiring oxygen at 1,000 to 3,000 Pa, (37), but cannot tolerate oxygen at the ordinary partial pressure in air (20,000 Pa). Lebek (21) differentiated *M. tuberculosis* from *Mycobacterium bovis* by the microaerophilic nature of the latter, and Grange and Yates (12) recommended this test to differentiate species within the *M. tuberculosis* complex. Marks (23) applied this approach in his identification scheme to differentiate aerobic *Mycobacterium* species (*M. tuberculosis*, *M. chelonae*, *M. flavescens*, and *M. fortuitum*) from microaerophilic species (*M. bovis*, *M. ulcerans*, *M. gordonae*, *M. terrae*, *M. avium*, and *M. intracellulare*). Respiratory assays on mycobacteria were performed during the early 1900s, mainly with *M. tuberculosis* (6, 8, 26, 36, 43). The investigators conducting these assays concluded that *M. tuberculosis* is strictly aerobic but that an atmosphere enriched with 5 to 10% CO_2 enhances growth, especially on agar media such as Middlebrook 7H10 (1). CO_2 seems to be essential for growth of mycobacteria. An appreciable proportion of carbon in mycobacteria arises from CO_2 fixation through a variety of carboxylation reactions (33). As Beam and Kubica reported, "It seems advisable to incubate all primary isolation cultures for mycobacteria in an atmosphere of 5–10% CO_2 in air" (1).

Our results show the microaerophilic nature of *M. genavense*, as shown by growth under the surface in semisolid medium, similar to *Campylobacter* sp. (20) and some other species of mycobacteria (e.g., *M. bovis*) described by Marks (23) in his identification scheme. We initially used 5% O_2 (10% CO_2 and 85% N_2) in the BACTEC system, the oxygen concentration commonly used for isolation of *Campylobacter jejuni* (20). Franzblau and Harris (11), however, demonstrated in a study of the metabolism of *M. leprae* that ATP maintenance is optimal at 2.5 to 10% O_2, and they proposed that a gas mixture of 2.5% O_2, 10% CO_2, and 87.5% N_2 could be used in the BACTEC system (10) for drug susceptibility testing. For *M. genavense*, earlier and better growth can be detected in the BACTEC system at low oxygen concentrations. Growth of the 12 different strains of *M. genavense* was detected with 2.5 or 5% oxygen within 6 weeks, even when bacillary concentrations were low. Conversely, at 21% oxygen, there was no detectable growth of 8 of the 12 strains (66.7%). The differences observed between the strains—some preferring 2.5% oxygen and others 5% oxygen—could not be correlated with the origins of the strains.

The decontaminated clinical specimen showed earlier growth of *M. genavense* in BACTEC vials gassed with low oxygen tension. In a comparison of the growth results following the different decontamination methods and incubation under low oxygen concentrations, better growth was obtained when the clinical material was decontaminated with NALC or SDS than by the Petroff method or HCl. At 2.5% oxygen, the GI reached 999 in 13 days with an inoculum of 10^6 AFB decontaminated with NALC or SDS; by comparison, only 10^4 AFB from nondecontaminated material were necessary to reach a GI of 999 in 13 days under the same gaseous conditions (Fig. 2). *M. genavense* appears to be very sensitive to decontamination procedures, and this may also explain the difficulties encountered in its in vitro growth.

In 1996, Hoop et al. (16) reported cultivation of *M. genavense* in 67.6% of bird tissues inoculated in BACTEC vials. It is important to note that the 32.4% negative cultures were inoculated with AFB-positive tissues by Ziehl-Neelsen staining. Moreover, these specimens were decontaminated by the SDS method (the same technique we used [35]) and inoculated in BACTEC vials supplemented with PANTA. We previously demonstrated that PANTA inhibits growth of *M. genavense*

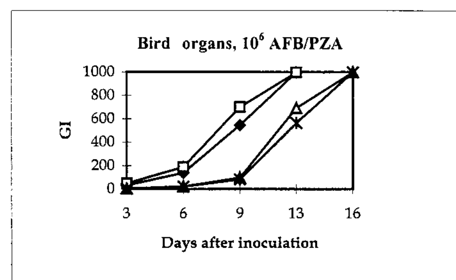

FIG. 5. Comparison of decontamination methods (SDS, NALC, Petroff, and HCl) for recovery of *M. genavense* from a clinical specimen in BACTEC vials gassed with 2.5% O_2, 10% CO_2, and 87.5% N_2. ◆, SDS; □, NALC; △, Petroff; ×, HCl.

(34). Although this was not specified by Hoop et al. (15, 16), we presume that the vials were gassed as recommended by the manufacturer—air (21% oxygen) with 5 to 10% CO_2. Among the factors affecting primary cultures of *M. genavense*, the medium, the decontamination method, and the oxygen tension have to be taken into account.

Recently, Wayne and Hayes (42) described experiments with cultures of *M. tuberculosis* in a deep liquid medium under conditions of known oxygen depletion. Two stages of nonreplicating persistence were observed, corresponding to microaerophilic and anaerobic conditions, possibly explaining the ability of *M. tuberculosis* to remain dormant in the host for long periods of time. By contrast, for atypical mycobacteria, which are not strict pathogens and are believed to have their reservoirs in the environment (29), we conjecture that microaerophilic conditions (2.5 or 5% O_2) do not lead to persistence but rather improve growth. As suggested by Jenkins et al. (17), factors such as aeration and carbon dioxide concentration may not have always been optimal for isolation of mycobacteria from the environment; in particular, *M. ulcerans* thus far has not been isolated from the environment despite numerous attempts (29, 30). As early as 1965, Hanks (13) proposed that it might be wise to conduct experiments at minimal concentrations of oxygen for cultivation of *M. leprae* and *Mycobacterium lepraemurium*, as these species universally produce nonpulmonary lesions.

Our experiments demonstrate the microaerophilic nature of *M. genavense* in primary cultures and suggest potential applications in routine laboratories which use the BACTEC system. Further studies are necessary to determine optimal carbon dioxide concentrations, as well as to test lower concentrations of oxygen. For isolation of atypical mycobacteria, e.g., *M. avium* or *M. ulcerans*, from clinical or environmental specimens, the possible microaerophilic nature of the organism should be taken into consideration, especially when difficult-to-grow species are suspected.

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