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Characteristics of an Unclassified *Mycobacterium* Species Isolated from Patients with Crohn's Disease

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The characteristics of an unclassified *Mycobacterium* sp. isolated from three patients with Crohn's disease are presented. The organism is extremely fastidious and mycobactin dependent and may require up to 18 months of incubation for primary isolation. Colony morphology is rough. Characteristics are unlike those of any presently defined species. The isolates produced positive niacin, catalase, and 2-week arylsulfatase reactions and were susceptible to neotetrazolium chloride (1:40,000), streptomycin (2 μg/ml), and rifampin (0.25 μg/ml). Chromogenicity, nitrate reduction, quantitative catalase, Tween hydrolysis, urease, tellurite reduction, pyrazinamidase, and 3-day arylsulfatase tests were negative, and the isolates were resistant to thiophene-2-carboxylic acid hydrazide (10 μg/ml) and isoniazid (10 μg/ml). Optimum growth in broth was determined to be in 7H9 medium with Dubos oleic albumin complex, Tween 80, and mycobactin J at 37°C without CO_2 or agitation and in low medium depth. This *Mycobacterium* sp. may be a subspecies or biovariant of *Mycobacterium paratuberculosis*, or it may represent a new species of *Mycobacterium*. It is suggested that this *Mycobacterium* sp. may play an etiological role in some cases of Crohn's disease.

Crohn's disease is a granulomatous form of enteritis which historically was noted for its resemblance to tuberculosis. The first accepted description of Crohn's disease appeared in the literature in 1932 (5). Although first described as a segmental disease of the small intestine, it was later recognized that the same disorder affected the colon and had been confused with ulcerative colitis (11). In more recent years, the lesions of Crohn's disease have been recognized in the mouth, larynx, esophagus, stomach, skin, muscle, synovial tissue, and bone (8). The histological hallmark of this disease is the presence of noncaseating epithelioid granuloma with Langhans-type giant cells, which occurs in 60% of patients (8).

During studies in which we sought an infectious agent in resected gastrointestinal tissues from patients with inflammatory bowel disease, we isolated strains of mycobacteria which had characteristics different from those of any presently recognized species (R. J. Chiodini, H. J. Van Kruiningen, W. R. Thayer, R. S. Merkal, and J. A. Coutu, Dig. Dis. Sci., in press). In this report we present the characteristics of these organisms.

MATERIALS AND METHODS

General. All media were supplemented with mycobactin (2 μg/ml) obtained from *Mycobacterium phlei* (mycobactin P) or *Mycobacterium paratuberculosis* (mycobactin J) prepared as follows (14). Roux bottles containing iron-deficient media were seeded with organisms which had been grown as a surface pellicle in liquid medium. After an appropriate time of incubation, the organisms were harvested and extracted with ethanol. The mycobactin was chelated with iron, extracted with chloroform, and purified by column chromatography. All cultures were incubated aerobically at 37°C without added CO_2.

Samples. Resected surgical specimens from patients with inflammatory bowel disease were examined. Specimens consisted of terminal ileum samples from 14 patients with Crohn's disease, colon samples from 6 patients with ulcerative colitis, and colon samples from 7 patients with cancer of the bowel. Immediately after surgery, the tissues were transported to the laboratory (56 miles) for processing. Tissues were washed in water, trimmed of fat, and stored at 3 to 5°C for 18 to 24 h in Butterfield buffer (1). The next day, ca. 10 g of mucosa was scraped from the surface and placed in sterile 2.5% trypsin (1:250 [pH 7.5]) with continuous stirring for 30 min. Tissues were filtered through cheesecloth and centrifuged at 4,340 × *g*, and the sediment was suspended in 40 ml of 0.1% benzalkonium chloride. After 18 to 24 h of decontamination, 0.1 to 0.2 ml of the resulting sediment was distributed onto each of eight tubes of Herrold egg yolk medium (HEYM). Tubes were incubated in a slanted position, with caps slightly loose, for 4 to 6 days to allow for evaporation of the inoculum. Caps were then sealed with paraffin, and the tubes were incubated in an upright position.

Media. Primary plating and isolation were performed on slants of fresh HEYM containing (per liter): 9.0 g of peptone, 4.5 g of NaCl, 15.3 g of Noble agar, 2.7 g of beef extract, 27.0 ml of glycerine, six egg yolks, 2.0 mg of mycobactin, 5.1 ml of malachite green (2%), and 870 ml of deionized distilled water. Broth cultures were grown in 30-ml (25-cm^2) tissue culture flasks containing 7 ml of Middlebrook 7H9 medium with oleic acid-albumin-glucose-catalase (OAGC), Tween 80, and mycobactin. Middlebrook 7H10 agar slants were prepared with OAGC and mycobactin in screw-capped tubes (25 by 125 cm). All media for biochemical testing were supplemented with mycobactin (2 μg/ml).

Tests. The arylsulfatase test was performed with a 3-day and a 2-week determination by the methods described by

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TABLE 1. Characteristics of *Mycobacterium* sp. isolated from patients with Crohn's disease

Characteristics[a]	Result[b]
Staining	Acid-fast bacilli
Size	1.8 to 2.3 μm by 0.3 μm
Colony morphology	Rough
Growth rate	
1° culture	15–72 weeks
Subculture	4–8 weeks
Mycobactin dependency	+
Growth on Lowenstein-Jensen media	−
Generation time (in liquid)	15–20 h
Chromogenicity	−
Growth	
8°C	−
22°C	Slight
30°C	Modest
37°C	Optimum
42°C	−
Niacin production	+ weak
Nitrate reduction	−
Catalase activity	
Room temperature	+
Quantitative	<45 mm
68°C	+
Tween 80 hydrolysis	−
Sodium chloride tolerance	−
Urease	−
Tellurite reduction	−
Pyrazinamidase activity	
4-day test	−
7-day test	−
Arylsulfatse activity	
3-day test	−
14-day test	+
Susceptibility to:	
TCH (10 μg/ml)	−
INH (10 μg/ml)	−
NT (1:40,000)	+
ST (2 μg/ml)	+
RIF (0.25 μg/ml)	+
Pathogenicity for[c]:	
Mice (IV, IP)	+
Mice (footpad)	−
Guinea pigs	−
Rabbits	−
Rats	−
Chickens	−
Goats	+

[a] Abbreviations: TCH, thiophene-2-carboxylic acid hydrazide; INH, isoniazid; NT, neotetrazolium chloride; ST, streptomycin; RIF, rifampin; IV, intravenous; and IP, intraperitoneal.
[b] Symbols: +, positive result; and −, negative result.
[b] Details published elsewhere (Chiodini et al., in press).

Kubica and Vestal (10). Substrate concentrations of phenolphthalein disulfate trisodium salt in Middlebrook 7H9 medium were 0.001 M for the 3-day test and 0.003 M for the 2-week test. Tubes containing 2 ml of substrate were inoculated with a turbid broth culture and incubated for 3 and 14 days. After an appropriate incubation period, 2 N sodium carbonate was added, and color change was compared to a set of standards. *Mycobacterium fortuitum* TMC 1529 was used as a positive control. Catalase activity was determined by the drop room temperature method at pH 7.0 and 68°C, and by the semiquantitative test (9). For the room temperature test, a spadeful of colonies from solid medium was transferred to a drop of Tween 80–30% hydrogen peroxide (1:1) and observed for the formation of bubbles. The pH 7.0, 68°C catalase test was performed by suspending colonies from solid media in 15 M phosphate buffer (pH 7.0) and heating at 68°C for 20 min. After cooling, Tween 80–30% hydrogen peroxide (1:1) was added, and the preparation was observed for the formation of bubbles. The semiquantitative catalase test was performed on a butt of solid medium which had been inoculated from broth culture. Tween 80-hydrogen peroxide (1 ml) was added, and gas formation was measured as the height of the column of bubbles above the media surface. Niacin production was determined by adding 0.5 ml of 4% aniline and 0.5 ml of 10% cyanogen bromide to 0.5 ml of an aqueous culture extract (16). A color change to yellow was regarded as a positive result. *Mycobacterium tuberculosis* TMC 201 was used as a positive control. Nitrite production was determined by suspending a spadeful of colonies in 0.1 M sodium nitrate (pH 7.0) and incubating the suspension at 37°C for 2 h, followed by the addition of 9 M HCl, 0.2% sulfanilamide, and 0.1% *n*-naphthyl-ethyleneamine dihydrochloride (20). A color change was compared to a set of standards. *M. tuberculosis* TMC 201 was used as a positive control. The pyrazinamidase test was performed by the 4- and 7-day method (21). Tubes of solid medium containing pyrazinamide were inoculated and incubated for 4 and 7 days. Ferrous ammonium sulfate was added to the tubes, which were then incubated at 3 to 5°C for 4 h. The development of a pink band in the agar was considered a positive reaction. *Mycobacterium intracellulare* TMC 1403 was used as a positive control. Tellurite reduction was detected in liquid media by the addition of 0.2% potassium tellurite and incubation for 3 days (7). *Mycobacterium avium* was used as a positive control. Tween 80 hydrolysis was determined after 24 h, 5 days, and 12 days (6). Tubes containing 0.5% Tween 80 and neutral red in phosphate buffer were inoculated from growth on solid media. A color change to pink was recorded as positive. *Mycobacterium kansasii* TMC 1201 was used as a positive control. Urease activity was detected with differentiation urea disks (Difco Laboratories, Detroit, Mich.). A urea disk was added to an inoculated tube containing 0.5 ml of sterile distilled water. A color change to red after 3 days was recorded as positive. *M. kansasii* TMC 1201 was used as a positive control. Sodium tolerance was determined on HEYM with 5% NaCl. Mycobactin dependency was determined by the failure to grow on HEYM without mycobactin

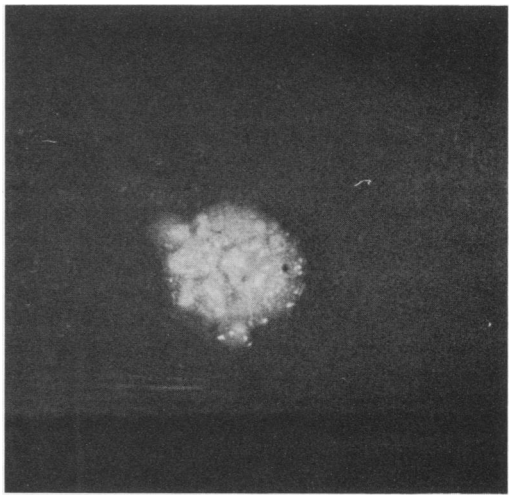

FIG. 1. Rough colony of *Mycobacterium* sp. grown on HEYM with mycobactin.

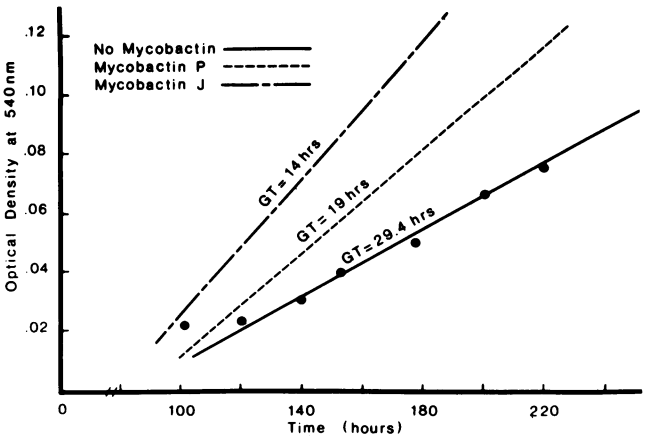

FIG. 2. Effects of mycobactin on growth rate of *Mycobacterium* sp. Optimum growth occurred on media supplemented with mycobactin J. GT, Generation time.

and commercially available Lowenstein-Jensen medium (BBL Microbiology Systems, Cockeysville, Md.).

Susceptibility to thiophene-2-carboxylic acid hydrazide (10 μg/ml), isoniazid (10 μg/ml), neotetrazolium chloride (1:40,000), streptomycin (2 μg/ml), and rifampin (0.25 μg/ml) was tested in tissue culture flasks containing 7H9 medium and appropriate drug concentrations. Each flask was inoculated with 50 μl of broth culture in stationary phase of growth. Drug susceptibility was determined by inhibition of growth as compared with control cultures inoculated with a 1:100 dilution of the organism into medium which did not contain antibiotics.

Serology and TLC. Serology and thin-layer chromatography (TLC) were performed on strains Linda and Dominic but not on strain Ben. Seroagglutination with antisera to serovariants of the *Mycobacterium avium-Mycobacterium intracellulare-Mycobacterium scrofulaceum* complex was performed by Anna Tsang of the National Jewish Hospital, Denver, Colo. Cultures grown in 7H9 broth were condensed and allowed to stand at room temperature for 48 h. Supernatant fluid containing a dispersed bacterial suspension was transferred and used in seroagglutination. Bacteria from ca. 100 ml of the supernatant 7H9 broth cultures were sedimented by centrifugation at 4,340 × *g* for 30 min and suspended in phosphate-buffered saline containing 0.5% Tween 80. Seroagglutination was performed with bacterial suspension in 7H9 broth and in phosphate-buffered saline with 0.5% Tween 80. Each bacterial suspension was adjusted with phosphate-buffered saline containing 0.5% phenol to an optical density of 0.3 at 525 nm. The seroagglutination procedure was performed by modification of the methods of Schaefer (17) as described previously (19). Agglutination was evaluated at 4 and 24 h after incubation at 37°C.

TLC was performed by P. J. Brennan of the National Jewish Hospital. Sediment from 7H9 broth, after standing for 48 h, was centrifuged at 4,340 × *g* for 30 min and used for TLC analysis as described previously (2, 19). Bacilli were extracted with chloroform-methanol, and the resulting extract was then treated with 9.2 M NaOH. Alkali-stable lipids were applied to silica gel TLC plates and chromatographed in chloroform-methanol-water at various concentrations.

Generation times were determined by an optical density method (22). Turbidity of broth cultures was recorded spectrophotometrically at 540 nm, and plate counts were obtained to determine the numbers of CFU at several optical densities. A graph was prepared plotting optical density at 540 nm versus CFU. Generation time was calculated by the change in hours of the optical density representing a single logarithmic growth phase (0.03 to 0.083) divided by the square root of 10 (3.3). The effects of Middlebrook OAGC enrichment, Middlebrook albumin-glucose-catalase enrichment, Dubos medium albumin, Dubos medium serum, Dubos oleic albumin complex (DOAC), Middlebrook 7H9 broth, and Dubos broth were determined and compared by generation time and overall growth rate. The effect of pyruvate was evaluated in HEYM and 7H9 broth at 4.1 g/liter.

Agitation effects on growth in broth cultures were determined on a rotary shaker at 50, 100, and 150 rpm. Tissue culture flasks containing 7 ml of medium were inoculated with 100 μl of a culture in stationary phase of growth, and optical density was recorded after 6 days of incubation. The effect of culture depth on growth was determined at medium depths of 2, 3, 4, 5, 6, 8, 10, 15, 20, 25, 30, and 40 mm. Tissue culture flasks were inoculated with 100 μl of a culture in stationary phase of growth, and optical densities were recorded daily to determine growth rates and generation times.

RESULTS

Three strains of aerobic, acid-fast rods with characteristics unlike those of previously described species were isolated from three patients with Crohn's disease. One isolate was obtained from a 15-year-old female (strain Linda), one from a 12-year-old male (strain Dominic), and the other from a 78-year-old male (strain Ben). Luxurious growth was not obtained on primary culture. Details of patient history will be published elsewhere (Chiodini et al., in press). These isolates belong to the genus *Mycobacterium* as presently defined (3), but they have characteristics unlike those of any described species. The characteristics of these isolates are summarized in Table 1.

Cultural characteristics. Primary colonies developed on HEYM after 3 (strain Linda), 5 (strain Dominic), and 18

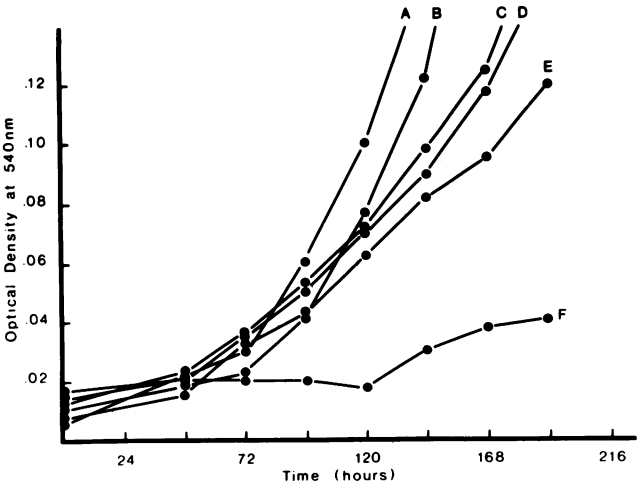

FIG. 3. Effects of medium enrichments on growth of *Mycobacterium* sp. Optimum growth occurred in 7H9 broth with DOAC. All media were supplemented with Tween 80 and mycobactin J. Media were 7H9 broth supplemented with DOAC (A), OAGC (B), albumin-glucose-catalase (C), Dubos medium serum (D), or Dubos medium albumin (E); and Dubos broth supplemented with DOAC (F).

(strain Ben) months of incubation. Colonies were 0.5 to 1.0 mm in diameter, brilliant white, irregularly shaped, mucoid, and rough (Fig. 1). Pigment was not produced. Subcultures grew in 4 to 6 weeks. Growth was more luxurious on media supplemented with mycobactin J than on media with mycobactin P. The organism failed to grow on Lowenstein-Jensen medium. Mycobactin dependency was not absolute; a few small colonies, visible with a dissecting microscope, developed on HEYM without mycobactin after ca. 16 weeks of incubation. Broth cultures grew in 2 to 4 weeks and appeared as white flakes (diameter, 1 to 3 mm) or as a diffuse granular suspension with sediment. Tween 80 or other dispersing agents were required; growth as a surface pellicle could not be obtained. Optimum temperature on HEYM was 37°C. Modest growth occurred at 30°C and slight growth occurred at 22°C. Colonies did not develop at 8 or 42°C. Growth was stimulated by the addition of pyruvate (4.1 g/liter) into HEYM. There was no effect on growth by incubation with CO_2.

Cells from HEYM and 7H9 broth were strongly acid-fast (99%) and measured 0.3 by 1.8 to 2.3 μm. Rods were straight or slightly curved. Staining was uniform or irregular, often showing beaded forms. Cells were arranged in clumps and there was some branching. Cords were not formed.

Biochemical reactions. All three strains had identical biochemical profiles. Tests for niacin were weakly positive. Several preparations were often necessary to detect the weak positive reaction. Catalase activity was demonstrable even after heating at 68°C for 20 min. Less than 45 mm of gas bubbles were produced in the semiquantitative test. Arylsulfatase was produced after 2 weeks. Growth was inhibited by media containing 5% NaCl. Nitrate and tellurite were not reduced. Pyrazinamide, urea, and Tween 80 were not hydrolyzed. In 7H9 broth with mycobactin, strains were susceptible to neotetrazolium chloride, streptomycin, and rifampin but not to isoniazid or thiophene-2-carboxylic acid hydrazide.

Serology and TLC analysis. All preparations of strain Linda were autoagglutinated after 4 h of incubation. Strain Dominic, when it was suspended in 0.05% Tween 80, agglutinated with a pair of type 42 antisera and one of a pair of types 13 and 18 antisera, whereas bacilli that were suspended in 7H9 broth agglutinated with a pair of type 4 antisera and one of a pair of types 43 and 3 antisera after incubation for 4 h. All preparations were autoagglutinated by 24 h.

TLC failed to reveal any specific lipid pattern useful for identification. Alkali treatment was not successful in eliminating nonspecific lipids which dominated the TLC pattern.

Growth characteristics. Under most conditions studied, strain Dominic had the fastest growth rate, and strain Ben had the slowest and most limited. Growth in 7H9 and 7H10 media was not as greatly affected by the incorporation of mycobactin, and dependency decreased with subculture. On third passage (HEYM to 7H9 broth to test medium), generation time in 7H9 broth without mycobactin was 32 h; with mycobactin P, it decreased to 24 h; and with mycobactin J, generation time was 14 h (Fig. 2). After multiple subcultures, generation time without mycobactin was reduced to 24 h, and with the incorporation of mycobactin P or mycobactin J, it remained at 14 to 20 h.

Middlebrook 7H9 broth was superior to Dubos broth in supporting growth of all strains. Maximum growth rates were obtained with 7H9 broth supplemented with DOAC, followed by 7H9 broth with OAGC (Fig. 3). Other supplements supported growth of a single strain better than the other strain (Table 2). Strain Dominic grew well in Dubos medium serum, reaching an optical density of >1 after 115 h, whereas strain Linda required 165 h, and strain Ben required 250 h. Compared with other strains, strain Ben grew best in Dubos medium albumin, reaching an optical density of >1 after 119 h; strains Linda and Dominic required 187 and 168 h, respectively. Organisms grew equally well in albumin-glucose-catalase. Contrary to the findings with HEYM, pyruvate inhibited growth in 7H9 medium.

TABLE 2. Generation times of unclassified *Mycobacterium* sp. in different growth supplements

Media, supplement[a]	Generation time (h) of *Mycobacterium* sp. strain:		
	Linda	Dominic	Ben
7H9, DOAC	10.33	10.3	14.5
7H9, OAGC	11.8	12.7	16.4
7H9, Dubos medium serum	25.2	10.9	29.6
7H9, Dubos medium albumin	22.4	21.8	21.2
7H9, albumin-glucose-catalase	17.0	21.5	27.0
Dubos broth, DOAC	NC[b]	NC	NC

[a] All media contained Tween 80 and mycobactin J.
[b] NC, Not calculated.

Medium depth had a great effect on growth rate, as determined by broth turbidity after 6 days of incubation (Fig. 4). Effects of depths below 1 mm could not be obtained because liquid was present in amounts insufficient for optical density determination. Maximum growth occurred at a medium depth of 1 mm. A rapid decline in growth occurred between depths of 1 and 6 mm; turbidity appeared to remain constant between 6 and 10 mm, and then declined until depths of 25 mm and higher, in which growth did not occur. This dependency on liquid depth did not appear to be related to aeration, since agitation during incubation greatly inhibited growth.

Optimum growth occurred in stationary cultures without agitation (Fig. 5). There was a marked decrease in broth turbidity when cultures were agitated at 50 rpm, a speed which caused little movement of the internal broth culture. Growth failed to occur at 100 and 150 rpm.

DISCUSSION

The species of mycobacteria described herein belong to the Runyon group III and appear to be related to the *M.*

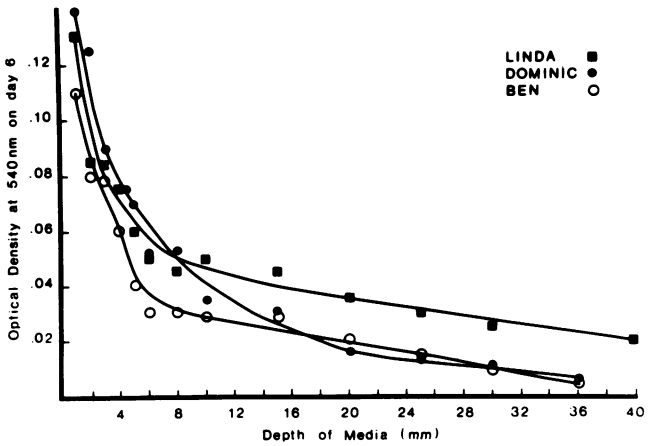

FIG. 4. Effects of medium depth on growth rate of *Mycobacterium* sp. Growth was most luxurious in low medium depth.

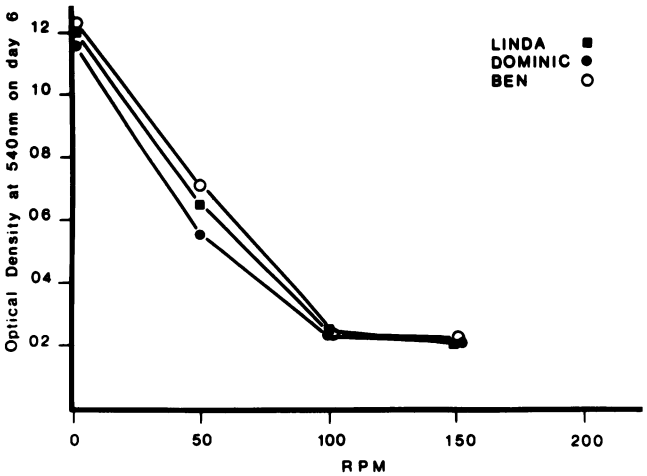

FIG. 5. Effects of agitation on growth of *Mycobacterium* sp. Agitation inhibited growth of all strains.

avium-M. intracellulare and *M. paratuberculosis* groups. The ever-increasing numbers of mycobactin-dependent mycobacteria, i.e., *M. paratuberculosis* (4), the "wood pigeon" bacillus (12, 18), and some strains of *M. avium* (13), have never been thoroughly examined, and identifications are based on only a few biochemical criteria. It has only been in recent times, in response to the mycobactin-dependent species of mycobacteria from patients with Crohn's disease, that the International Working Group on Mycobacterial Taxonomy (IWGMT) has considered examination of the mycobactin-dependent group of mycobacteria. It will, however, be several years before this group of organisms will be taxonomically characterized.

The mycobacteria isolated from patients with Crohn's disease are very similar to *M. paratuberculosis* in both cultural and biochemical characteristics (22). Biochemically, differences occur only in arylsulfatase and niacin reactions. As with *M. paratuberculosis*, these organisms are mycobactin dependent, and they grow best at low medium depth in Middlebrook 7H9 broth supplemented with DOAC and in stationary cultures without added CO_2 (22). Despite these similarities, the National Veterinary Services Laboratory (Ames, Iowa) and a reference laboratory for the IWGMT failed to identify the organisms. The mycobacteria described herein and *M. paratuberculosis* also have many antigenic similarities as shown by the enzyme-linked immunosorbent assay method (W. R. Thayer, J. A. Coutu, R. J. Chiodini, H. J. Van Kruiningen, and R. S. Merkal, Dig. Dis. Sci., in press). Although thorough antigenic comparisons have not been made, it has been observed that antibodies produced against strains Linda and Dominic react more strongly with these strains than with *M. paratuberculosis*. Serology and TLC analysis were not helpful in characterizing the mycobacteria from patients with Crohn's disease. Autoagglutination occurred in all serotyping schemes, and TLC analysis failed to identify specific lipid patterns useful for identification, a characteristic also shared with *M. paratuberculosis*.

There is little uncertainty that these mycobacteria are not members of any well-defined species and apparently belong to the poorly characterized mycobactin-dependent group. Further studies are required to determine if these organisms represent a new species of *Mycobacterium* or a biovariant or subspecies of *M. paratuberculosis*. Final taxonomic characterization must await decisions from the IWGMT.

An infectious etiological agent of Crohn's disease has been sought since the first description of the disease appeared in the literature in 1932 (5). None has been found. All candidate agents, including viruses and a host of different bacteria, have not withstood independent investigations and have been disregarded in recent years (8). The remarkable resemblance of Crohn's disease to intestinal tuberculosis has always been recognized (15, 23); however, the failure to isolate *M. tuberculosis* and the inability to identify acid-fast bacilli in tissue sections from patients with Crohn's disease has caused the dismissal of mycobacteria as etiological agents. Nevertheless, over the years, recurrences of the notion that Crohn's disease might have a mycobacterial etiology have persisted.

The successful isolation of a pathogenic *Mycobacterium* sp. from some patients with Crohn's disease has revived the idea of a mycobacterial etiology. The *Mycobacterium* sp. isolated produces a noncaseating granulomatous ileocolitis in goats (Chiodini et al., in press), and the immunological data have shown that a significant proportion ($P = 0.0003$) of patients with Crohn's disease recognize mycobacterial antigens as measured by the enzyme-linked immunosorbent assay (Thayer et al., in press). These findings suggest that the *Mycobacterium* sp. reported herein may play a role in some cases of Crohn's disease. The fastidiousness of this organism, its mycobactin dependency, the long incubation periods required for growth, and the special concentration techniques used for isolation may explain why this organism has not been reported previously from patients with Crohn's disease.

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