

Growth and Metabolic Characteristics of *Mycobacterium paratuberculosis*

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The cultural characteristics of newly isolated strains of *Mycobacterium paratuberculosis* on a variety of media were studied. The mycobactin dependence ascribed to this species of *Mycobacterium* was found to be easily circumvented by incorporation of 1% ferric ammonium citrate in serum or egg yolk media, but the earliest and most abundant growth occurred on serum medium that contained mycobactin and sodium pyruvate. In the presence of ferric ammonium citrate, respiration did not decrease during 5 days; but in the presence of all other agents studied, respiration decreased after the first day.

Mycobacterium paratuberculosis, the etiologic agent in Johne's disease, is considered by most investigators to be extremely difficult to culture and, therefore, is seldom included in studies of mycobacteria. *M. paratuberculosis* is usually isolated on serum or egg yolk medium supplemented with mycobactin. Mycobactin dependence and slow growth are the chief characteristics used to distinguish newly isolated *M. paratuberculosis* from other mycobacteria with similar colony appearance such as members of the *Mycobacterium avium* complex. Strains of *M. paratuberculosis* maintained in the laboratory frequently lose mycobactin dependence, no longer require complex media, and grow more rapidly than newly isolated strains.

This paper describes the cultural characteristics of newly isolated strains of *M. paratuberculosis* when grown on a variety of media frequently used to culture mycobacteria and the effects of alterations in these basic media on growth of *M. paratuberculosis*. The effects of metabolic intermediates and growth stimulators on oxygen consumption of the organisms are described.

MATERIALS AND METHODS

Organisms. Forty-four strains of *M. paratuberculosis* isolated from infected animals in 14 herds of cattle and 1 herd of goats were used. All strains initially were isolated on mycobactin-egg yolk medium by the benzalkonium chloride method (2). Each strain was subcultured on egg yolk medium with and without mycobactin; those that grew only on egg yolk medium containing mycobactin were considered to be *M. paratuberculosis*.

Media. The basic media used were: egg yolk agar

(2), serum agar (4), Watson-Reid agar (3), and Dorset-Henley synthetic medium with agar (1) prepared as shown in Table 1, plus 7H10 agar and Dubos broth with agar and oleic-albumin complex (Difco Laboratories, Detroit, Mich.). The basic media were supplemented as indicated under Results with one or more of the following: ferric mycobactin (*Mycobacterium phlei* derived, 2 mg/liter), ferric ammonium citrate (10 g/liter), sodium pyruvate (4.1 g/liter), albumin (10 g/liter), and oleic acid-albumin-dextrose-catalase complex (OADC, Difco) (100 ml/liter). All media were dispensed in 25-mm² (30-ml) plastic tissue culture flasks. Each medium was inoculated with 0.5 ml of a 0.5% Tween-80 suspension of the organisms and incubated at 37 C. Growth was assessed visually and recorded after 5 and again after 10 weeks of incubation.

Respiration. Organisms to be used in respirometry studies were harvested from the medium and washed three times by repeated suspension, centrifugation, and resuspension of the sediment in saline. The washed suspension of organisms was standardized by centrifugation of a sample of the suspension in a Hopkins tube (5). Conventional manometric methods at 37 C in air were used, except that observation periods were sometimes extended for as long as 5 days and aseptic techniques were used throughout to prevent contamination.

RESULTS

Culturing. Colony morphology. There were no apparent differences between strains of *M. paratuberculosis* due to their primary herd source. The primary colonies on mycobactin-egg yolk medium were small, smooth, moist, convex, and nonpigmented. When first subcultured from mycobactin-egg yolk medium, the colonies on mycobactin-serum agar appeared the same as those on mycobactin-egg yolk medium.

TABLE 1. Comparison of amounts of ingredients in various basic media

Ingredients	Basic media (g/liter except where otherwise indicated)						
	Egg yolk	Serum agar	Watson-Reid	Dorset-Henley	Dubos broth	7H10	7H10-OADC
Sodium citrate		1.5		0.9		0.4	0.4
Ammonium citrate		2.0					
Ferric citrate				0.3			
Ferric ammonium citrate (green)			0.075		0.05		
Ferric ammonium sulfate						0.04	0.04
Sodium chloride	4.5		2.0		0.17		0.85
Potassium phosphate (monobasic)		1.0	2.0		1.0	1.5	1.5
Sodium phosphate (dibasic)		2.5		1.42	2.5	1.5	1.5
Magnesium sulfate		0.6	1.0	1.5	0.01	0.025	0.025
Zinc sulfate			0.01	0.027	0.0001	0.001	0.001
Ammonium sulfate						0.5	0.5
Copper sulfate				0.004	0.0001	0.001	0.001
Calcium chloride			0.02	0.067		0.0005	0.0005
Cobalt nitrate				0.0013			
Cobalt chloride			0.002		0.0005		
Pyridoxine hydrochloride						0.001	0.001
Biotin						0.0005	0.0005
Catalase							0.004
Beef extract	2.7						
Peptone	9.0						
Bacto-Casitone or Casamino Acids		2.5			0.5		
Asparagine		0.3	5.0	14.0	2.0		
L-Glutamic acid						0.5	0.5
Glucose			10.0	10.0			2.0
Glycerol	34.0	31.0	60.0	63.0	5.0	5.0	5.0
Bovine fraction V albumin					10.0		5.0
Bovine serum		200.0 ml					
Chicken egg yolk	120.0 ml						
Tween 80		0.5			0.2		
Oleic acid							0.05
Agar	15.3	15.0	15.0	15.0 ^a	15.0 ^a	15.0	15.0

^a Added when solid medium was required.

On mycobactin-7H10-OADC, there were a variety of colony types. The predominant colony type was small, moist, and convex; but some colonies were multi-domed, some were circular with a concave surface, and some were flat. Older colonies developed a hollow space between the center of the colony and the surface of the agar. Further subcultures of each colony type on mycobactin-7H10-OADC produced a higher proportion of the parent type, but pure cultures of any single colony type could not be maintained. The colony types discernible on 7H10-OADC medium were much more difficult to recognize on the other media.

A comparison of quantity and speed of growth of newly isolated strains is given in Table 2.

Ferric ion. All strains were "mycobactin dependent" when first isolated in the sense that they did not grow on egg yolk, serum, or

7H10-OADC medium with the usual amounts of iron and without mycobactin. The chicken egg yolk used provides 8 to 9 mg of iron per liter of medium and bovine serum provides approximately 0.3 mg of iron per liter. However, all strains grew without mycobactin when these media were supplemented with 1% ferric ammonium citrate. The growth frequently was slower than when mycobactin was present, but the total growth, after 10 weeks of incubation, usually was as great on medium with 1% ferric ammonium citrate as with mycobactin.

Repeated subcultures on serum agar did not alter the requirement for mycobactin or ferric ammonium citrate when subcultures were grown on serum or egg yolk agars; but later subcultures maintained on mycobactin serum agar grew on 7H10-OADC without either mycobactin or ferric ammonium citrate; and with

TABLE 2. Comparison of amounts of total growth of newly isolated *M. paratuberculosis* on various media supplemented as indicated^a

Supplement ^b	Basic medium						
	Egg yolk agar	Serum agar	Watson-Reid	Dorset-Henley	Dubos broth	7H10	7H10-OADC
None	0	0	0	0	0	0	0
FM	3(8)	4(8)	1(12)	1(12)	4(10)	2(8)	2(8)
FAC	3(10)	4(10)	0	0	4(10)	2(12)	3(8)
P	0	0	0	0	0	0	0
FM + P	3(7)	4(7)	1(12)	1(12)	4(9)	2(9)	3(7)
FAC + P (H)	0	0	0	0	0	0	0
FAC + P (S)	3(9)	4(9)	0	0	4(9)	2(9)	3(8)

^a First numbers indicate maximum growth, graded 0 to 4; numbers in parentheses indicate incubation time, in weeks, when maximum growth occurred.

^b FM, Ferric mycobactin (2 mg/liter); FAC, ferric ammonium citrate (10 g/liter); P, sodium pyruvate (4.1 g/liter); (H), supplements heated together, either in the medium or before being added to the cooled medium; (S), supplements heated separately before being added to the cooled medium.

each subsequent subcultivation, growth on 7H10-OADC was greater.

After one passage in Dubos broth with ferric ammonium citrate, the organisms grew well on 7H10-OADC.

Pyruvate. The addition of sodium pyruvate to mycobactin-serum agar stimulated more rapid growth of most strains of *M. paratuberculosis*. When organisms were grown on mycobactin-7H10-OADC medium with pyruvate, the colonies which appeared earliest were on subsequent culture, shown to be stimulated by pyruvate. When the organisms were grown on mycobactin-7H10-OADC without pyruvate, many colonies were found which later were shown to be inhibited by pyruvate.

Pyruvate-ferric ammonium citrate antagonism. Growth was stimulated when pyruvate was added before autoclaving to the serum or egg yolk media that contained mycobactin, but growth was inhibited when pyruvate was added before autoclaving to media that contained ferric ammonium citrate. Growth was stimulated when ferric ammonium citrate and pyruvate were autoclaved separately and then either combined or added separately to cold media by flooding them on the surface and allowing them to diffuse into the media. When they were autoclaved together before they were added to media, no growth occurred.

The comparatively greater stimulatory effect of mycobactin than of ferric ammonium citrate was temporary; i.e., visible and maximum growth occurred about 2 weeks earlier on media that contained mycobactin than on media that contained ferric ammonium citrate; but after 10 weeks of incubation, there usually was no observable difference. Likewise, growth occurred 1 to 2 weeks earlier on mycobactin-serum agar

supplemented with pyruvate than without pyruvate, but the final amount of growth was usually equal. In contrast, subcultures on 7H10-OADC selected for pyruvate stimulation still had many more and larger colonies after 10 weeks of incubation in the presence of pyruvate, whereas the subcultures selected for pyruvate inhibition grew well only in the absence of pyruvate.

Albumin. After three to five subcultures on serum agar with mycobactin, most strains grew moderately on 7H10-OADC without mycobactin or ferric ammonium citrate. Growth on 7H10 medium supplemented with oleic acid, dextrose, and catalase, but without albumin, was equivalent to that on the medium containing complete OADC. Adding albumin to Dorset-Henley or Watson-Reid media did not materially improve them. Hence, albumin did not seem to be an important medium ingredient for *M. paratuberculosis*.

Variant selection. Cultures maintained on mycobactin-supplemented serum or egg yolk media rarely grew on Watson-Reid or Dorset-Henley media; but after cultivation on 7H10-OADC, variants sometimes were obtained which grew on both Watson-Reid and Dorset-Henley media. Colonies which grew were very rough and easily adapted to slow growth as a pellicle on synthetic liquid medium. 7H10 medium supplemented only with glycerol supported the growth of only a few variants, and these also grew on Watson-Reid or Dorset-Henley media.

Respirometry. The organisms initially consumed 44% more oxygen in the presence of 1% ferric ammonium citrate than in the presence of ammonium citrate and 41% more than in the presence of 0.025 M succinate. Oxygen con-

sumption was 21% ($0.4 \mu 10_2$ per ml per h) more than endogenous respiration in the presence of succinate. Oxygen consumption due to mycobactin was not detected.

Endogenous oxygen consumption and oxygen consumption for all metabolites were relatively higher for organisms grown with ferric ammonium citrate than for those grown with mycobactin. The ratio of oxygen consumed in endogenous respiration to that consumed in the presence of metabolites was the same for organisms grown with ferric ammonium citrate and organisms grown with mycobactin. The oxygen consumption per hour per unit weight of organisms in the presence of ferric ammonium citrate was the same after 5 days as during the first day, whereas oxygen consumption per hour per unit of organisms in the presence of all other metabolites and during endogenous respiration decreased as time increased.

When organisms were grown on serum agar containing mycobactin and not selected for growth stimulation by pyruvate, less oxygen was consumed in the presence of 0.015 M pyruvate in saline than in saline alone during the first 8 h. At 72 h, respiration was equal in saline alone and saline that contained pyruvate; and by 96 h, respiration in the flasks containing pyruvate was 47% higher than endogenous. When Dubos broth was used instead of saline, initial respiration was equal in the presence or absence of pyruvate; and at all later times, it was greater in the presence of pyruvate. In contrast, respiration of the organisms in the presence of 0.012 M acetate in saline was slightly stimulated during the first few hours and thereafter was always less than in saline alone; but when Dubos broth was used instead of saline, respiration was always 25 to 30% higher in the presence of acetate.

DISCUSSION

The fastest and most abundant early growth of newly isolated *M. paratuberculosis* was on serum agar containing mycobactin. Most strains were further stimulated by pyruvate, which apparently stimulated growth by reducing the need for a highly active glycolytic pathway.

Part of "mycobactin dependence" of newly isolated strains of *M. paratuberculosis* on complex media such as egg yolk or serum agars seems to reflect an inability of the organisms to

extract sufficient ferric ion from the media. Other mycobacteria produce mycobactins that sequester the iron, but *M. paratuberculosis* does not produce sufficient mycobactin for this purpose. The addition of exceptionally high levels of ferric ammonium citrate saturates any iron-binding capacity of the egg yolk or serum and leaves a surplus of ferric ion available to the organisms.

Egg yolk medium is commonly used for isolation of *M. paratuberculosis* because the egg yolk neutralizes the benzalkonium chloride used to decontaminate fecal or intestinal tissue specimens. Serum agar also is frequently used, but more laborious decontamination procedures then are required. Neither of these media, however, allows the expression of colony morphology differences that are readily demonstrated on 7H10 media.

Apparently, the ferric ions present in the organisms when harvested were gradually dissipated into the medium during respirometry studies, so that oxygen consumption by the organisms decreased with time when each of the metabolites was studied, as well as during tests of endogenous respiration. However, ferric ion as supplied by ferric ammonium citrate allowed respiration to continue at a maximum rate.

After *M. paratuberculosis* is isolated from infected animals on mycobactin-egg yolk or mycobactin-serum media, it can be readily cultivated on a variety of media. During the first several transfers, a high level of ferric ion must be in the media; otherwise, the cultivation of this organism is not greatly different from that of other mycobacteria, and it should be included in appropriate comparative studies.

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