

## Culture Phenotypes of Genomically and Geographically Diverse *Mycobacterium avium* subsp. *paratuberculosis* Isolates from Different Hosts<sup>∇</sup>

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*Mycobacterium avium* subsp. *paratuberculosis* causes paratuberculosis (Johne's disease) in ruminants in most countries. Historical data suggest substantial differences in culturability of *M. avium* subsp. *paratuberculosis* isolates from small ruminants and cattle; however, a systematic comparison of culture media and isolates from different countries and hosts has not been undertaken. Here, 35 field isolates from the United States, Spain, Northern Ireland, and Australia were propagated in Bactec 12B medium and Middlebrook 7H10 agar, genomically characterized, and subcultured to Lowenstein-Jensen (LJ), Herrold's egg yolk (HEY), modified Middlebrook 7H10, Middlebrook 7H11, and Watson-Reid (WR) agars, all with and without mycobactin J and some with sodium pyruvate. Fourteen genotypes of *M. avium* subsp. *paratuberculosis* were represented as determined by BstEII IS900 and IS1311 restriction fragment length polymorphism analysis. There was no correlation between genotype and overall culturability, although most S strains tended to grow poorly on HEY agar. Pyruvate was inhibitory to some isolates. All strains grew on modified Middlebrook 7H10 agar but more slowly and less prolifically on LJ agar. Mycobactin J was required for growth on all media except 7H11 agar, but growth was improved by the addition of mycobactin J to 7H11 agar. WR agar supported the growth of few isolates. The differences in growth of *M. avium* subsp. *paratuberculosis* that have historically been reported in diverse settings have been strongly influenced by the type of culture medium used. When an optimal culture medium, such as modified Middlebrook 7H10 agar, is used, very little difference between the growth phenotypes of diverse strains of *M. avium* subsp. *paratuberculosis* was observed. This optimal medium is recommended to remove bias in the isolation and cultivation of *M. avium* subsp. *paratuberculosis*.

*Mycobacterium avium* subsp. *paratuberculosis* is the causative agent of Johne's disease of ruminants, which occurs in most countries. Usually it is an enteric infection with a long incubation period and progressively worsening granulomatous infiltration of the small and large intestines leading to weight loss and death. It is economically significant in farmed cattle, goats, sheep, and deer and is known to be capable of infecting a wide range of domesticated and free-living herbivores and occasionally also the carnivores that prey upon them (2, 4, 6, 15). Like many pathogenic mycobacteria, *M. avium* subsp. *paratuberculosis* is not host specific, and cross-species transmission is common where there are high contact rates. Although tissue colonization and histological lesions may occur, clinical disease in response to infection is rarely reported for nonruminant species, except for rabbits (2, 4). There is a long-standing debate about the role of *M. avium* subsp. *paratuberculosis* in chronic inflammatory bowel disease of humans, specifically Crohn's disease. Consequently, the isolation and cultivation of *M. avium* subsp. *paratuberculosis* are attempted in both veterinary

and medical diagnostic and research laboratories worldwide (20, 33, 46).

Isolation of *M. avium* subsp. *paratuberculosis* from feces, which is the most relevant and common clinical sample, is not straightforward because the organism grows extremely slowly and cultures are prone to contamination. A primary requirement for *in vitro* culture of *M. avium* subsp. *paratuberculosis* is inclusion of mycobactin in media to enable iron acquisition; mycobactin dependence is used as a discriminatory test for confirmation of *M. avium* subsp. *paratuberculosis* in cultures. Apart from these considerations, *M. avium* subsp. *paratuberculosis* has been cultivated on a wide range of media since its original isolation in 1910 (46).

*M. avium* subsp. *paratuberculosis* was once believed to be a relatively homogenous taxon with 98% identity to *Mycobacterium avium* subsp. *avium* (serovars 2 and 5) based on comparisons by DNA hybridization (27–28). However, the discovery of the multicopy IS900 element (14) and its use as a probe in restriction fragment length polymorphism (RFLP) analysis revealed more than 20 genotypes of *M. avium* subsp. *paratuberculosis* in screens of isolates gathered from around the world. The patterns were due to mutations at restriction endonuclease cut sites and variation in the numbers of copies and the sites of insertion in the genome of IS900. Two primary finger-

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print patterns were identified, leading to classification of isolates as S strains (derived from sheep), C strains (derived from cattle and other species), and intermediate strains (9); these are also known as type I, II, and III strains, respectively (12, 39). Immediately, a correlation was noticed between strain type and ease of isolation, as the S strains were notoriously difficult to grow. Greater genetic diversity was later identified, particularly between S and C strains, as small and large genomic deletions and insertions and single nucleotide polymorphisms were found (13, 24–26). The so-called S and C strains probably represent the extremes of a taxon; a continuum may exist, and this will be determined when representative genomes have been sequenced and annotated. Cross-species transmission under sufficiently high contact rates can cloud this basic separation (50). Of interest in the context of culture is the so-called *M. avium* subsp. *paratuberculosis* bison (B) strain. This was identified using *IS1311* polymorphism analysis of isolates from one population of bison in the United States, and it too is difficult to isolate from clinical samples (17, 45, 49).

At the phenotypic level, isolates of *M. avium* subsp. *paratuberculosis* can be distinguished by several criteria, including the degree of pigmentation of colonies (rare orange-pigmented versus common nonpigmented varieties) (39–40), culturability (S strains are not easily cultivable, but C strains are more readily cultivable), and degree of clumping of cells in saline suspensions (35). While *M. avium* subsp. *paratuberculosis* is defined as being mycobactin dependent during *in vitro* cultivation (42, 44), Spanish researchers reported that *M. avium* subsp. *paratuberculosis* from clinical cases of paratuberculosis in sheep could be isolated on some media without inclusion of mycobactin (1). Although this property was observed using Middlebrook 7H11 medium, the isolates required mycobactin for growth on Lowenstein-Jensen (LJ) medium. It remains unclear whether these observations were due to idiosyncrasy in the types of media used or the growth capacities of the particular strains. Although there is evidence for a beneficial effect of sodium pyruvate (18, 29), it is not always included in culture media and may be inhibitory to some strains (19).

Despite the economic importance of *M. avium* subsp. *paratuberculosis* in livestock and its potential role in Crohn's disease in humans, there has never been a systematic international comparison to determine the suitability of culture media for different strains of this organism or consideration of possible geographic variants of *M. avium* subsp. *paratuberculosis* with different cultural requirements. Consequently, the aims of this study were to investigate phenotypic differences among isolates from different species and countries, to correlate phenotypic characteristics with genotypic characteristics, and to evaluate the suitability of media for cultivation of *M. avium* subsp. *paratuberculosis*. Specifically, is there a universally applicable culture medium?

#### MATERIALS AND METHODS

**Isolates of *M. avium* subsp. *paratuberculosis*.** To obtain a diverse range of *M. avium* subsp. *paratuberculosis* isolates, a collection was compiled from four widely separated geographic regions, among which there had been no recent animal host contact: Australia, Northern Ireland (United Kingdom), Spain, and the United States. Isolates were obtained for this study as cultures on either Herrold's egg yolk medium with mycobactin J (HEY+MJ), modified Middle-

brook 7H10 medium with MJ (7H10+MJ), or Middlebrook 7H9 broth (Table 1), following their primary isolation on HEY+MJ (Australia, United States, Northern Ireland, Spain), Lowenstein-Jensen medium (Spain), or modified Middlebrook 7H9-based media (Bactec) (Australia) (Table 1). Alternatively, some isolates that had been isolated originally in this way were lyophilized and stored; they were reconstituted in 0.5 ml of sterile water. All isolates were considered to be field isolates and had not been subjected to repeated laboratory subcultures. A volume of 0.1 ml of colony suspension, broth, or reconstituted lyophilized suspension was inoculated into modified Bactec 12B with mycobactin J medium and incubated at 37°C, and the growth index (GI) was measured weekly. By 4 weeks, the GI for all isolates was >400 and a 0.1-ml aliquot was taken and inoculated onto modified 7H10+MJ slopes, which were incubated at 37°C. After 6 weeks, there was very heavy growth of tiny colonies on all slopes. From each slope, a suspension was prepared from multiple colonies by dragging a plastic microbiology loop over a linear field 1 cm in length and emulsifying the colonies so obtained in sterile saline with 0.5% (vol/vol) Tween 80 (ST) (29). These undiluted suspensions were mixed thoroughly. A 50-fold dilution of each suspension was made in ST. The identity of each isolate as *M. avium* subsp. *paratuberculosis* was confirmed by examination of a smear stained with Ziehl-Neelsen stain and by PCR and restriction endonuclease analysis of the *IS900* and *IS1311* elements (see below).

**Culture media and inoculation.** Each isolate was subcultured onto 14 different solid media (Table 2). The media were prepared in 35-ml polycarbonate culture tubes (Techno-Plas; catalog no. C8027SU) using 10 to 11 ml medium per tube, which was allowed to form a slant.

Watson-Reid agar, with the addition of ammonium citrate, was prepared according to the method of Merkal and Curran (29) and consisted of (per liter) L-asparagine (5 g), D-glucose (10 g), glycerol (63 ml), ammonium hydrogen citrate (2 g), ferric ammonium citrate (75 mg), potassium dihydrogen phosphate (2 g), sodium chloride (2 g), magnesium sulfate (1 g), zinc sulfate (10 mg), calcium chloride (20 mg), cobalt chloride (2 mg), Noble agar (1 g; Difco), and, if required, mycobactin J (2 mg; Allied Monitor). The pH was adjusted to 5.5 with 1 M NaOH. The medium was autoclaved at 121°C for 20 min.

Lowenstein-Jensen medium contained (per liter) potassium dihydrogen phosphate (1.5 g), magnesium sulfate (310 mg), magnesium citrate (375 mg), L-asparagine (2.25 g), malachite green (12.5 mg), homogenized whole egg (625 ml), and, if required, sodium pyruvate (4 g) and mycobactin J (2 mg). The medium was both sterilized and set in the culture tubes by steaming them at 85°C for 30 min.

Herrold's egg yolk medium was prepared by the method of Merkal and Curran (29) and contained (per liter) peptone (9 g; Difco, BD), sodium chloride (4.5 g), Noble agar (15.3 g), beef extract (2.7 g; Oxoid), glycerol (27 ml), malachite green (100 mg), egg yolk (120 ml), and, if required, mycobactin J (2 mg). The medium was autoclaved prior to the addition of egg yolk and, if required, sodium pyruvate (4 g/liter). No antibiotics were added to this medium.

Middlebrook 7H10 medium was based on that of Whittington et al. (48) and contained (per liter) Middlebrook 7H10 agar (14.3 g; Difco), Casitone (750 mg; Difco), glycerol (3.76 ml), an antibiotic mixture (polymyxin B at 37,600 U, amphotericin B at 3.8 mg, nalidixic acid at 15 mg, trimethoprim at 3.8 mg, azlocillin at 3.8 mg, and polyoxyethylene stearate at 150 mg [Panta Plus]; Becton Dickinson), a supplement (bovine albumin at 3.8 g, dextrose at 1.5 g, and catalase at 3 mg [ADC enrichment]; Difco), egg yolk (188 ml), and, if required, mycobactin J (940 µg). The antibiotic mixture, supplement, egg yolk, and mycobactin J were added after the medium was autoclaved.

Middlebrook 7H11 medium was based on that of Whittington et al. (48) and contained (per liter) Middlebrook 7H11 agar (14.3 g; Difco), glycerol (3.76 ml), an antibiotic mixture (polymyxin B at 37,600 U, amphotericin B at 3.8 mg, nalidixic acid at 15 mg, trimethoprim at 3.8 mg, azlocillin at 3.8 mg, polyoxyethylene stearate at 150 mg [Panta Plus]; Becton Dickinson), a supplement (oleic acid at 37.6 mg, bovine albumin at 3.8 g, dextrose at 1.5 g, catalase at 3 mg [OADC]; Difco), and, if required, mycobactin J (940 µg). The antibiotic mixture, supplement, and mycobactin J were added after the medium was autoclaved.

A volume of 40 µl of each undiluted and diluted bacterial suspension was inoculated at the top of the slope and allowed to run down the surface and to spread out. Cultures were incubated at 37°C. Each tube was inspected weekly for 14 weeks under bright transverse illumination using magnification. The time to appearance of colonies was recorded, as was colony size. Colonies were called minute where they were clearly visible only with magnification and pinpoint where tiny colonies were observed with the naked eye; the colony diameter was estimated when colonies were large enough to be observed easily with the naked eye.

**Extraction and purification of DNA.** DNA extraction was performed as described by Choy et al. (7). Briefly, 0.4- to 0.5-g (wet weight) bacterial cell pellets

TABLE 1. Source of 35 *M. avium* subsp. *paratuberculosis* isolates and one *M. avium* subsp. *avium* isolate, their cultural characteristics, and molecular characterization<sup>h</sup>

Isolate	Accession no. <sup>a</sup>	Primary medium <sup>b</sup>	Form <sup>c</sup>	Country	Isolate source	Incubation period (wk), colony size on indicated culture medium <sup>d</sup>				
						WR+MJ	LJ+MJ	LJ+P+MJ	HEY+MJ	HEY+P+MJ
4	Telford 9-2	Bactec	SL	Australia	Sheep feces		13, m	14, m		
5	Telford 9-2	Bactec	FD	Australia	Sheep feces		13, m	13, m		
6	VRS1065 (Vanderstock)	HEY+MJ	SL	Australia	Sheep		13, m	13, m	6, p	11, p
7	VRS1068 (Wrigley)	HEY+MJ	SL	Australia	Sheep		13, m	13, m	7, m	7, m
8	FD1104 (Sharwood)	HEY+MJ	SL	Australia	Sheep			3, m	6, p	8, p
12	99/145-10	Bactec	SL	Australia	Alpaca feces			13, m		
13	99/454-10	Bactec	SL	Australia	Sheep feces					
23	98/91-1	Bactec	FD	Australia	Llama		13, m	11, m	6, p	6, p
24	Telford poly	Bactec	SL	Australia	Sheep feces		13, m	10, m		
25	98/101	Bactec	SL	Australia	Nematode larvae		13, m	11, m		
26	PC98/86-1	Bactec	SL	Australia	Sheep feces		13, m	3, m	11, m	
27	116/1 (Parfett)	Bactec	SL	Australia	Cow feces		13, m	9, m	11, m	
35	CM00/416	HEY+MJ	SL	Australia	Cow lymph node		13, m	3, m	7, p	7, p
36	CM00/426	HEY+MJ	SL	Australia	Cow feces		13, m	13, m	7, p	7, p
20	99/21-1	HEY+MJ	SL	Northern Ireland	Sheep feces	9, p	13, m	9, m	6, p	7, p
21	99/21-34	HEY+MJ	SL	Northern Ireland	Sheep feces		13, m	3, m	7, p	7 (1)
22	99/21-G5	HEY+MJ	FD	Northern Ireland	Sheep milk		13, m	13, m	6, p	6, p
2	JD98/107-3 (938i ovi)	LJ+MJ	BR	Spain	Sheep		13, m	13, m	6, p	4, m
1	JD98/107-1 (17G ovi)	LJ+MJ	BR	Spain	Sheep		13, m	13, m	12, p	
3	JD98/107-6 (22G ovi)	LJ+MJ	BR	Spain	Sheep		13, m	13, m	7, p	13, p
9	99/233 (191)	LJ+MJ	BR	Spain	Sheep		13, m	13, m	12, p	
10	99/233 (181)	LJ+MJ	BR	Spain	Sheep		13, m	13, m	8, m	
14	99/87-78 (2)	HEY+MJ	FD	United States	Bison	2 (1)	2	2, p	1	3 (4)
15	99/87-87 (4)	HEY+MJ	FD	United States	Bison		13, m	3, m	7, p	7, p
16	99/87-33 (10)	HEY+MJ	FD	United States	Goat		13, m	13, m	7, p	6, p
17	99/87-91 (14)	HEY+MJ	FD	United States	Cow		13, m	3, m	7, p	7, p
18	99/87-174 (18)	HEY+MJ	FD	United States	Cow		13, m	3, m	7, p	7, p
34	JD99/87-36 (30)	HEY+MJ	FD	United States	Bison		13, m		7, p	10, m
31	JD00/41-1	HEY+MJ	SL	United States	Gibbon		13, m		7, p	7, p
32	JD00/41-12	HEY+MJ	FD	United States	Tule elk		13, m	3, m	7, p	7, p
33	JD00/41-13	HEY+MJ	FD	United States	Elk			13, m	9, p	10, p
39	JD00/41-21	HEY+MJ	SL	United States	Springbok		13, m	13, m	7, p	7, m
40	JD00/41-24	HEY+MJ	SL	United States	Tule elk		13, m	13, m	6, m	6, m
41	JD00/41-25	HEY+MJ	SL	United States	Oryx		13, m	3, m	7, p	7, m
42	JD00/41-14	HEY+MJ	SL	United States	Elk		13, m	3, m	7, p	8, p
43	JD00/41-26	HEY+MJ	SL	United States	Springbok		13, m	8, m	6, p	6, p

<sup>a</sup> Original accession names/codes are provided in parentheses.

<sup>b</sup> The medium used for primary isolation from the clinical sample. Abbreviations are as described in Table 2, but medium constituents may vary from those in the present study and also between laboratories.

<sup>c</sup> Cultures were supplied as slopes (SL), as broth cultures in Middlebrook 7H9 medium (BR), or as freeze-dried ampoules (FD).

<sup>d</sup> Incubation period in weeks for the appearance of colonies and the maximum colony size recorded at 14 weeks, as follows: p, pinpoint colonies visible to the naked eye; m, minute colonies visible only with a hand lens; and number in parentheses, diameter of a representative colony in millimeters. WR, Watson-Reid agar; P, sodium pyruvate.

<sup>e</sup> MP, *M. avium* subsp. *paratuberculosis*; Neg, negative.

<sup>f</sup> A, *M. avium*; B, bison strain; C, cattle strain; S, sheep strain.

<sup>g</sup> The RFLP profiles are profiled in Fig. 1 and 2.

<sup>h</sup> Isolates are grouped by country of origin.

were resuspended in 1 ml of 10 mM Tris, 1 mM EDTA, pH 8.0 (TE), after which DNA extraction was undertaken using lysozyme and mutanolysin in conjunction with proteinase K. Following this, cetyltrimethylammonium bromide (CTAB) and a standard chloroform-isoamyl alcohol technique was used to purify the DNA. The purified DNA was resuspended in sterile TE (pH 8.0) and stored at 4°C. The concentration of the DNA was calculated by spectrophotometry (Pharmacia; GeneQuant II) using the formula ( $\mu\text{g/ml} = A_{260} \times \text{DF} \times 50$ ), where DF is the dilution factor.

**PCR and restriction endonuclease analysis.** IS900 and IS1311 PCR and restriction endonuclease analyses were performed as described previously to confirm the identities of isolates of *M. avium* subsp. *paratuberculosis* and to classify them as C, S, or B strains (10, 23, 30).

**IS900 and IS1311 RFLP analysis.** Substrain determination was undertaken by restriction fragment length polymorphism (RFLP) analysis using probes for IS900 and IS1311 according to the methods described previously (7–8). Briefly, the DNA sample was digested with the restriction endonuclease BstEII (Roche). The digested DNA samples were separated on 1% (wt/vol) agarose gels by electrophoresis at 35 V for 20 h and transferred under vacuum (Hybaid) to positively charged nylon membranes (Amersham). A 229-bp IS900 PCR-based digoxigenin-labeled probe was hybridized with the membrane overnight. After being washed, the membrane was then developed with a digoxigenin chemiluminescence kit (Roche) and exposed to X-ray film for 2 h. The films were then developed using standard procedures. At the completion of the IS900 RFLP process, the

membranes were stripped and reprobated using a 268-bp IS1311 digoxigenin-labeled probe (23). The RFLP process was completed as described above.

The IS900 RFLP profiles identified in this study were compared with published profiles (8, 31, 34, 47). The IS1311 RFLP profiles identified in this study were also compared with published RFLP profiles (8). However, in the absence of a naming system in that study, one is proposed here: B for bovine profiles, O for ovine profiles, and In for intermediate profiles (In is used to differentiate this profile from IS900 RFLP intermediate [I] profiles).

**Microscopic appearance.** The morphology of the isolates was assessed by preparing smears from a saline suspension made from colonies grown on HEY medium plus MJ (HEY+MJ) and Middlebrook 7H10 medium plus MJ (7H10+MJ) for 8 weeks. Smears were stained by a Ziehl-Neelsen method and examined at a  $\times 1,000$  magnification using oil immersion.

**Ease of suspension of colonies in saline.** A sample of several colonies was taken using a bacteriological loop and mixed with 20  $\mu\text{l}$  of saline on a glass microscope slide to form a suspension. The suspension was examined with the naked eye using bright illumination to assess its uniformity.

## RESULTS

**Identification and genomic characterization of isolates.** After exclusion of four contaminated cultures, all but one of the

TABLE 1—Continued

		Incubation period (wk), colony size on indicated culture medium <sup>d</sup>						PCR-REA result with probe for:		RFLP <sup>e</sup> with probe for:		
7H10+MJ	7H11+MJ	WR	LJ	LJ+P	HEY	HEY+P	7H10	7H11	IS900 <sup>e</sup>	IS1311 <sup>f</sup>	IS900	IS1311
4 (1)	2, p							10, m	MP	S	S1	O1
4, m	2, m							7, m	MP	S	S1	O1
3, p	2, m							7, m	MP	S	S1	O1
3, m	2, m							7, m	MP	S	S1	O1
3, p	2, m							7, m	MP	S	S1	O2
8 (1)	6, p							7, m	MP	S	S1	O1
6 (1)	5, p							7, m	MP	S	S1	O1
3, p	2, p							2, m	MP	C	C3	B1
3, p	2, p							5, m	MP	S	S1	O1
3, p	2, p							2, m	MP	S	S1	O1
3, p	2, p							2, m	MP	S	S1	O1
3, p	2, p							5, m	MP	S	S1	O1
3, p	2, p							5, p	MP	C	C3	B1
3, p	2, m							2, p	MP	C	CU8	B1
3, p	2, p							5 (3)	MP	C	C5	B1
3, p	2, p							2, p	MP	C	CU7	B1
3 (4)	2 (1)							5, p	MP	C	C5	B1
3, p	2, p							2, m	MP	C	CU5	B1
4, p	3, p							14, m	MP	S	I8	O3
4, m	5, p							6, p	MP	S	I9	O1
4 (1)	5 (0.5)							6, p	MP	S	I10	O3
4 (1)	5 (2)							6 (0.5)	MP	S	I11	In1
1 (1)	2, p	2	2	2	1	3	1	2, p	Neg	A		
3, p	2, p							2, m	MP	B	C1	B1
3, p	3, p			13, m				8, p	MP	C	CU6	B1
3, p	2, p							2, p	MP	C	C1	B1
3, p	2, p							2, p	MP	C	CU4	B1
3, p	2, p							5, m	MP	B	C5	B1
3, p	2, p							12, m	MP	C	C1	B1
3, p	2, p							2, p	MP	C	C3	B1
6 (1)	2 (0.5)							2, m	MP	C	C1	B1
3, p	2, p							2, m	MP	C	C1	B1
3, p	2, p							6, m	MP	C	C3	B1
3, p	2, p							6, m	MP	C	C1	B1
3, p	2, p							2, m	MP	C	C1	B1
3, p	2, p							2, m	MP	C	C1	B1

remaining 36 field isolates were identified as *M. avium* subsp. *paratuberculosis*; the single isolate that was not confirmed to be *M. avium* subsp. *paratuberculosis* by IS900 PCR and restriction endonuclease analysis (REA) originated from a bison in the United States and produced an *M. avium* subsp. *avium* profile in the IS1311 PCR-REA (isolate 14 in Table 1). It was retained in the study as a control, as it would likely grow on all media.

The strain type of *M. avium* subsp. *paratuberculosis* was established using IS1311 PCR-REA. The majority of the IS1311 types matched those expected based on the host species of

origin, with the following exceptions. Three isolates originating from sheep samples from Northern Ireland produced C profiles, as did a single isolate from a sheep sample from Spain. Of the 35 field isolates of *M. avium* subsp. *paratuberculosis*, there were 18 C, 15 S, and 2 B isolates (Table 1). The C isolates were derived from cattle, sheep, goats, a primate, wild ruminants, and a camelid. The S isolates were derived from cattle, sheep, and a camelid, while the B isolates were from bison only.

RFLP analysis was used to subtype the isolates. Four previously unknown BstEII IS900 RFLP profiles were identified

TABLE 2. Culture media used in this study

Medium	Base medium	Abbreviation	Egg	MJ concn	Additive(s)
1	Herrold's egg yolk	HEY+MJ+P	Yolk	2 mg/liter	Sodium pyruvate
2	Herrold's egg yolk	HEY+P	Yolk	Nil	Sodium pyruvate
3	Herrold's egg yolk	HEY+MJ	Yolk	2 mg/liter	Nil
4	Herrold's egg yolk	HEY	Yolk	Nil	Nil
5	Lowenstein-Jensen	LJ+MJ+P	Whole	2 mg/liter	Sodium pyruvate
6	Lowenstein-Jensen	LJ+P	Whole	Nil	Sodium pyruvate
7	Lowenstein-Jensen	LJ+MJ	Whole	2 mg/liter	Nil
8	Lowenstein-Jensen	LJ	Whole	Nil	Nil
9	Middlebrook 7H11	7H11+MJ	Nil	940 µg/liter	Oleic acid, albumin, dextrose, catalase
10	Middlebrook 7H11	7H11	Nil	Nil	Oleic acid, albumin, dextrose, catalase
11	Watson-Reid (pH 5.5)	WR+MJ	Nil	2 mg/liter	Nil
12	Watson-Reid (pH 5.5)	WR	Nil	Nil	Nil
13	Middlebrook 7H10	7H10+MJ	Yolk	940 µg/liter	Albumin, dextrose, catalase
14	Middlebrook 7H10	7H10	Yolk	Nil	Albumin, dextrose, catalase



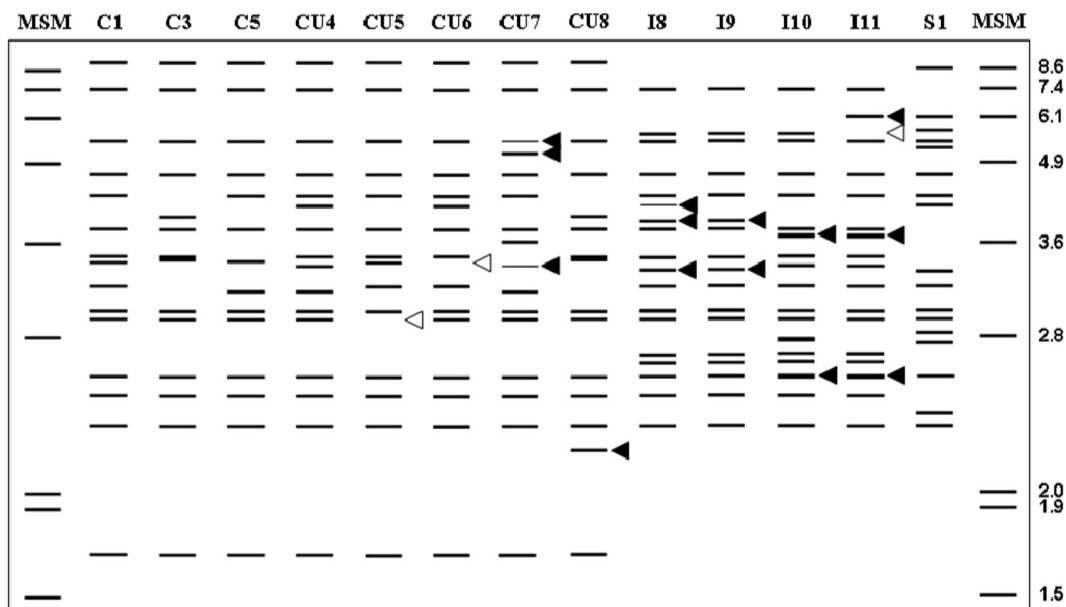


FIG. 1. *IS900* RFLP profiles observed in this study. New profiles are characterized by differing band intensities and the presence (◄) and/or absence (<) of bands responsible for the new profile. MSM, molecular size markers. Numbers at the right are molecular sizes (in kilobase pairs).

(Fig. 1). By following the nomenclature of Whittington et al. (47), these were named CU5, CU6, CU7, and CU8 and originated from Spain, the United States, Northern Ireland, and Australia, respectively. All four profiles correlated with C strain profiles generated from the *IS1311* PCR-REA typing. C1 profiles dominated the cattle types, and all of the *IS900* RFLP C subtypes had the same pattern, B1, with the *IS1311* probe. The genomic DNA of bison (B) strains subtyped by *IS1311* PCR-REA as C1-B1 and C5-B1 with *IS900* and *IS1311* probes.

No new *IS900* S strain RFLP profiles were observed; all had S1 profiles, but they divided into two subtypes (S1-O1 and S1-O2) with the *IS1311* probe.

Four new *IS900* RFLP intermediate (I) profiles were observed; all were from sheep samples originating from Spain. By following the most recent identification of new I profiles generated from BstEII *IS900* RFLP typing (31), these were named I8 to I11. One of these, I11, had a unique *IS1311* RFLP profile, the first to be described as an intermediate profile (In1). The profile lacked the uppermost band that is typical of *IS1311* RFLP S profiles and the lowermost band that is typical of the *IS1311* RFLP B profiles (Fig. 2).

Based on RFLP typing with the *IS900* and *IS1311* probes, there were a total of 14 genotypes of *M. avium* subsp. *paratuberculosis*: eight subtypes of C isolates (profiles C1-B1, C3-B1, C5-B1, CU4-B1, CU5-B1, CU6-B1, CU7-B1, and CU8-B1), two subtypes of S isolates (S1-O1 and S1-O2), and four subtypes of I isolates (I8-O3, I9-O1, I10-O3, and I11-In1) (Table 1).

**Growth of *M. avium* subsp. *paratuberculosis* on a range of culture media.** The isolates of *M. avium* subsp. *paratuberculosis* grew satisfactorily on a narrow range of media (Table 3). Middlebrook 7H10 and 7H11 media with mycobactin J (MJ) supported the growth of all isolates, most commonly by week 2 or 3, and the most common colony size was pinpoint. More isolates had colonies with a diameter of >0.5 mm on Middle-

brook 7H10 medium than on any other medium. With the exception of Middlebrook 7H11 medium, MJ was an essential component of all media for the cultivation of field isolates of *M. avium* subsp. *paratuberculosis*. On Middlebrook 7H11 medium without MJ, colonies of all 35 field isolates were apparent in 2 to 14 weeks, but almost all were minute even after 14 weeks of incubation, which is not optimal growth (Table 3). The addition of MJ to this medium improved the growth of *M. avium* subsp. *paratuberculosis*, as the colonies appeared sooner in many cases and were often larger.

Lowenstein-Jensen medium (LJ) with MJ and with or with-

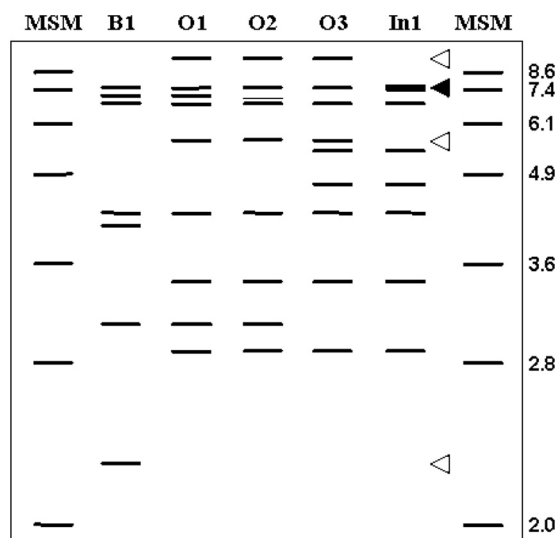


FIG. 2. *IS1311* RFLP profiles observed in this study. New profiles are characterized by the presence (◄) and absence (<) of bands responsible for the new profile. Numbers at the right are molecular sizes (in kilobase pairs).

TABLE 3. Patterns of growth of 35 field isolates of *M. avium* subsp. *paratuberculosis* on media supporting growth

Medium <sup>a</sup>	Abbreviation	No. of field isolates that grew	No. of wks to appearance of colonies <sup>b</sup>	Colony size <sup>a</sup>	Comment(s) on type of isolates
1	HEY+P+MJ	23	4-11 (7)	Minute to 1 mm (pinpoint)	Isolates which failed to grow were S strain isolates
3	HEY+MJ	29	6-12 (7)	Minute to pinpoint (pinpoint)	Isolates which failed to grow were S strain isolates
5	LJ+P+MJ	32	3-14 (13)	Minute to pinpoint (minute)	
6	LJ+P	1	13	Minute	Isolate 16, United States, goat, C strain
7	LJ+MJ	30	13	Minute	
9	7H11+MJ	35	2-6 (2)	Minute to 2 mm (pinpoint)	
10	7H11	35	2-14 (2)	Minute to 3 mm (minute)	
11	WR+MJ	1	9	Pinpoint	Isolate 20, Northern Ireland, sheep feces, C strain
13	7H10+MJ	35	3-8 (3)	Minute to 4 mm (pinpoint)	

<sup>a</sup> Numbers correspond to numbers in Table 2.

<sup>b</sup> Data are ranges (modes).

out sodium pyruvate supported the growth of many isolates, but most required prolonged incubation to yield only minute colonies. The isolates that did not grow on these media were mostly S strains.

Herrold's egg yolk medium with MJ (HEY+MJ) supported the growth of most isolates. Colonies appeared usually at about 7 weeks and, for most isolates, were pinpoint in size. The isolates that did not grow were all S strains. However, some S strains did grow on this medium, including several from Australia that were originally recovered from sheep before 1986 and one from cattle, as well as several of Spanish origin. More isolates grew on HEY+MJ without sodium pyruvate than on HEY+MJ with sodium pyruvate.

Watson-Reid medium with MJ was unsuitable for cultivation of field isolates of *M. avium* subsp. *paratuberculosis*, suggesting that it is suitable only for laboratory-adapted strains. Only one field isolate grew on this medium.

The colony morphologies of *M. avium* subsp. *paratuberculosis* isolates did not vary greatly between isolates or media. All isolates formed small domed colonies with a shiny surface and entire edges on all media on which they grew. However, there was a difference between the colors of colonies grown on HEY+MJ and 7H10+MJ. Colonies were mostly white on HEY+MJ. On 7H10+MJ, colonies were initially cream colored, but after 7 to 10 weeks, the colonies of some isolates became white. On 7H11+MJ, they were translucent, white, or occasionally yellow.

*M. avium* subsp. *avium* (isolate 14) grew on all media and had cream-colored colonies which became yellow after about week 6.

**Ziehl-Neelsen staining observations.** The morphologies of the isolates in smears stained by a Ziehl-Neelsen method were similar for colonies grown on HEY+MJ and 7H10+MJ for most isolates: short acid-fast bacilli. However, morphologies differed between media for C strain isolates 2, 3, 20, 21, 23, 31, 32, 33, 34, 35, and 36 and S strain isolate 3. For these isolates, the bacteria grown on 7H10+MJ appeared shorter and wider than those grown on HEY+MJ. On the latter medium, there were sometimes relatively long, slender bacilli.

**Ease of emulsification of colonies in saline.** When mixed with a bacteriological loop in a drop of saline on a glass slide,

all isolates grown on 7H10+MJ were able to be suspended in saline. In contrast, isolates that grew on HEY+MJ tended to remain in small but macroscopically visible clumps when the bacteria were mixed with saline on a slide.

### DISCUSSION

The primary finding of this study with respect to the suitability of culture media for the growth of diverse *M. avium* subsp. *paratuberculosis* isolates was that media based on Middlebrook 7H9 agar supported the growth of all isolates. None of the other media that were evaluated did this, and none resulted in such large colonies (the most common size was pinpoint colonies visible to the naked eye) within reasonable incubation periods. MJ was an essential component for growth on Middlebrook 7H10 agar and greatly improved growth on Middlebrook 7H11 agar; that is, MJ was not required for growth on egg-free Middlebrook 7H11 medium but did stimulate growth. All of the isolates evaluated were propagated first in Bactec medium and then in 7H10+MJ to obtain uniform suspensions for inoculation of solid media. Thus, it could be argued that there was selection bias toward isolates that grew best on such media. In fact, except for the Australian isolates, which were derived mostly from primary Bactec 12B (Middlebrook 7H9) broth cultures, the isolates had negative selection bias, as most isolates were originally recovered from clinical samples using HEY+MJ or LJ+MJ (Spain), and all of these retained their ability to grow on these media (Table 2).

Testing for mycobactin dependency is a fundamental method for identification of *M. avium* subsp. *paratuberculosis*. The data presented here suggest that it is possible to conduct this test using microbial suspensions grown on Middlebrook 7H10 agar, HEY medium, or LJ medium. Strict protocols have been suggested for determination of mycobactin dependency due to attachment of mycobactin from culture media to bacteria prior to their subculture to mycobactin-free medium (21). Although some carryover of mycobactin was possible from the 7H10+MJ that was used to prepare suspensions in this study, it did not appear to be of practical significance, because none of the isolates grew on Middlebrook 7H10 medium or HEY medium unless mycobactin was included and only one grew on

LJ medium. An important observation from this study is that mycobactin dependency should not be assessed on egg-free Middlebrook 7H11 medium.

Sodium pyruvate was reported to stimulate the growth of some isolates of *M. avium* subsp. *paratuberculosis* in HEY+MJ or LJ+MJ, compensating for the inhibitory effects of antibiotics that were included in the latter medium (18, 29). Inclusion of pyruvate may have been based on findings from culture of the tubercle bacillus (22). However, when included in HEY+MJ in this study, three S strain isolates from sheep from Spain failed to grow. These results were consistent with original reports from Spain in which pyruvate appeared to stimulate the growth of isolates of *M. avium* subsp. *paratuberculosis* from cattle but inhibited the isolation of *M. avium* subsp. *paratuberculosis* from sheep and goats (19).

Consistent with findings of previous studies, S strains from various sources grew poorly on HEY+MJ. The exceptions were S strains from Spain and three S strain isolates from sheep in Australia, which were cultured from clinical samples in the early 1980s, lyophilized, and archived (isolates 6, 7, and 8 in Table 1). Apart from these isolates, there are no known S strains from Australia with the capacity to grow on HEY+MJ; in fact, until the development of media suitable for the cultivation of S strains in 1998 (48), a presumptive diagnosis of paratuberculosis due to the S strain was made when cultures on HEY+MJ from animals that had typical histopathological lesions of paratuberculosis were negative. It is unknown why these isolates from the very early stages of the paratuberculosis epizootic in sheep in Australia (37) and not isolates obtained later in the epizootic can grow on HEY+MJ. It is possible that there was no selective advantage in the biochemistry underlying this growth capacity, and it was lost during microbial evolution in the sheep population. Alternatively, the alternative phenotype had a selective advantage *in vivo* and now predominates. Genomic studies are warranted to compare isolates 6, 7, and 8 with later isolates, such as isolate 4, which has been more fully characterized (24) to determine the genetic basis of the growth differences.

The growth of all five Spanish isolates on HEY media is not consistent with previous results from Spain, where only a small proportion of isolates from sheep grew on this type of medium (19). Possibly the isolates used in this study were not representative of those used in the earlier study, or the HEY media used in the two laboratories differed in some important way. Additionally, it might be possible that after primary isolation on LJ+MJ, which was common practice in Spain when the isolates were obtained, subculture on other egg-based types of media is facilitated. In a separate study in Spain, tissue homogenates from cattle and goats yielded colonies of *M. avium* subsp. *paratuberculosis* on a range of media, but only C strains grew readily on HEY media (11).

Lowenstein-Jensen medium has been popular for the routine isolation of *M. avium* subsp. *paratuberculosis* from clinical samples in northern Europe for decades (18). However, in studies of sheep paratuberculosis in other parts of Europe, colonies on this medium were tiny and took longer to grow than on Middlebrook 7H11 agar (1). These findings were confirmed in this study and were independent of strain type; overall, LJ medium did not support the growth of *M. avium* subsp. *paratuberculosis* as well as other media did.

Watson-Reid medium is a chemically defined medium with a high iron level, and there is one report suggesting that at low pH, mycobactin is not required for cultivation of *M. avium* subsp. *paratuberculosis* in this medium (32). However, as found in the present study, others have reported that *M. avium* subsp. *paratuberculosis* does not grow well on this medium (29, 43).

Fourteen genotypes of *M. avium* subsp. *paratuberculosis* originating from a range of species from four geographically separated countries were included in this study, thereby providing a robust assessment of *M. avium* subsp. *paratuberculosis* diversity in the context of culturability. Differences in culturability were broadly defined in terms of IS1311 PCR-REA genotype, in that S strains tended to grow poorly on media except Middlebrook 7H9 and related solid media (7H10, 7H11) with mycobactin. The few Australian S strains which grew on HEY+MJ were not distinguished by RFLP analysis from ones which did not grow on this medium, while the S strains from Spain had unique RFLP profiles. Collectively, these data suggest that RFLP profiles alone are not predictive of growth requirements. Exceptions exist to the general rule of thumb that S strains do not grow on HEY+MJ. Therefore, there must be another genomic feature which is correlated with but not linked to IS900 loci. Furthermore, although there have been many reports that show an association between host ruminant species, isolate type, and culturability (3, 9, 19, 38), the host ruminant species does not determine culturability. For example, isolation of S strains from cattle was difficult (50), isolation of C strains from several bison populations was easily accomplished, and isolation of B strains from the original infected herd and later from a dairy cow was mostly unsuccessful on HEY+MJ (17).

A prior observation that, compared to colonies of an S strain, colonies of the laboratory-adapted C strain 316V grown on 7H10+MJ were difficult to emulsify in saline (35) does not extend to field strains of *M. avium* subsp. *paratuberculosis*. C strain 316V has other properties which differ from those of field strains, such as the capacity to grow on Watson-Reid medium (unpublished data). In the present study, the ease of emulsification between S and C field strains grown on 7H10+MJ was less different than between C field strains grown on HEY+MJ and S field strains grown on 7H10+MJ. The tendency to clump appears to be a feature of the culture medium upon which the organisms are grown rather than the strain of *M. avium* subsp. *paratuberculosis per se*. Presumably, organisms grown on these two media utilize different growth factors and have different cell surface characteristics as a result. Differences observed in cell morphology for some isolates grown on different media were interesting but require quantification in a future study.

The rate of growth of *M. avium* subsp. *paratuberculosis* is an important practical consideration in diagnostic laboratories and should be assessed when selecting a culture medium for routine use. It is well recognized that incubation periods for some solid media often need to be prolonged; for example, 70% of S strain isolates took more than 3 months to grow, and 50% took 5 to 7 months on LJ or Middlebrook 7H11 medium (11). The results of the present study indicate that very prolonged incubation periods can be avoided by use of a more suitable medium: the interval to the appearance of colonies on



7H10+MJ was 3 weeks (mode) compared to 13 weeks on LJ+MJ.

It may be unwise to make direct comparisons between data presented here for certain culture media and those in previous studies because media with the same name may not be formulated identically across studies; the compositions of media were often not described in earlier studies. To avoid problems with future interpretation of the data in this study, the culture media are described in detail herein. Importantly, the 7H10+MJ used in this study contained egg yolk, a modification introduced specifically to enable the culture of S strains (48). While modified 7H10+MJ may be a universally applicable culture medium for *M. avium* subsp. *paratuberculosis*, for diagnostic applications which require greater analytical sensitivity, liquid culture media with a Middlebrook 7H9 base are recommended (46).

In summary, the differences in growth that have been reported historically among species of hosts and *M. avium* subsp. *paratuberculosis* strain types, particularly the difficulty of culture of *M. avium* subsp. *paratuberculosis* from sheep and sometimes other small ruminants in diverse settings, such as Australia (5), New Zealand (9), England (41), Iceland (16), Spain (11, 19), and the United States (36), have been strongly determined by the type of culture medium used. When using an optimal culture medium, such as modified 7H10+MJ, there is very little difference between growth phenotypes of S, C, and B strains of *M. avium* subsp. *paratuberculosis*. Since one solid medium formulation supported the growth of all isolates in this study, it is logical for diagnosticians to request the use of this medium alongside other solid media that may be in routine use to maximize the chances of isolation of *M. avium* subsp. *paratuberculosis*. This optimal medium is recommended to remove bias in the isolation and cultivation of *M. avium* subsp. *paratuberculosis*.

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