Differentiation Among Mycobacterial Species by Thin-Layer Chromatography

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Thin-layer chromatography of the ethyl ether-ethanol-soluble fraction after incubation with [^{35}S]methionine is useful for differentiation among mycobacterial species. Scanning of the radioactivity on chromatograms showed various distribution patterns of radioactive spots, and the patterns were, in most cases, characteristic for the species.

Analysis of lipid composition is known as a useful tool for differentiating mycobacterial species (9, 10, 13, 14, 16, 17, 21, 22, 24, 33). Radioisotope techniques have not yet been used for differentiation of mycobacteria, except by Tsukamura et al. (31), who measured the uptake of [^{35}S]methionine in various mycobacteria. They measured the radioactivity of whole cells after incubation with [^{35}S]methionine and observed that pathogenic group III mycobacteria (*Mycobacterium avium* and *M. intracellulare*) and *M. tuberculosis* and *M. bovis* showed significantly lower activity than the other mycobacteria. The present study was designed to develop the previous method into a more reliable one.

MATERIALS AND METHODS

The strains used are listed in Table 1. Most of the strains were identified through cooperative studies of the International Working Group on Mycobacterial Taxonomy (IWGMT) (11, 18, 35). Some strains were identified in this laboratory by methods described previously (29, 30). The organisms were grown in Ogawa egg medium at 37 C for 5 days (rapidly growing mycobacteria, *M. phlei* through *M. flavescens* in Table 1) or 14 days (slowly growing mycobacteria, *M. kansasii* through *M. goodii* in Table 1). The cells were washed three times in saline and then weighed; 100-mg amounts (wet weight) of the cells were suspended in 2.0 ml of 0.067 M phosphate buffer solution (pH 7.1) containing 5 μCi of L-[^{35}S]methionine and 10 μg of sodium acetate per ml. The tubes containing the mixture were stoppered with cotton plugs and incubated at 37 C for 20 h. After incubation, the cells were centrifuged at 500 $\times g$ for 15 min and washed twice with 5.0 ml of chilled water. The cells were extracted twice with 2.0 ml of 10% trichloroacetic acid, each for 10 min. The acid extracts were separated from the cells by centrifugation and discarded. The cells were then extracted twice with 3.0 ml of ethyl ether-ethanol (1:1, vol/vol) for 10 min each. During the first 5 min of the extraction, the cells were mixed continuously with a glass stick at room temperature (25 $\pm$ 3 C); during the second 5 min, the tubes

were allowed to stand in an incubator at 37 C, during which time the cells sedimented. The extracts could then be separated easily by means of a pipette. The combined ethyl ether-ethanol-soluble fractions were labeled as "lipid fraction." The residue was dissolved in 2.0 ml of a 1% NaOH solution by heating at 100 C in a water bath for 5 min.

L-[^{35}S]methionine was a product of New England Nuclear Corp., Boston, Mass. (specific activity, 264 Ci/mmol; radiochemical purity, >93%; containing <7.0% methionine sulfoxide, <0.2% methionine sulfone, and <0.2% homocysteic acid). Ethyl ether was a product of Katayama Chemical Company, Osaka, Japan (specific gravity, below 0.720; fraction [34 to 35 C], minimum 95% [by volume]; water, maximum 0.3%; nonvolatile, maximum 0.002%; acidity [CH_3COOH], maximum 0.003%). Ethyl alcohol also was a product of Katayama Chemical Company, Osaka, Japan (bp, 78 C; specific gravity, below 0.797; nonvolatile, maximum 0.003%; acidity [CH_3COOH], maximum 0.003%; heavy metals [Pb], maximum 0.0002%; ketones [$\text{CH}_3\text{CO}\cdot\text{CH}_3$], maximum 0.002%; assay by specific gravity, minimum 99.46% [by volume]). Samples, each 0.2 ml, were taken from the lipid fraction and the residue fraction.

The samples were placed in stainless-steel planchets and dried by heating with an infrared lamp. The radioactivity of each sample was measured by a gas flow counter. The samples of the lipid fraction and the residue fraction contained the radioactivity from 3.3 mg (wet weight) and 10 mg (wet weight) of the cells, respectively.

After the sample was taken for measurement of radioactivity, the remaining lipid fraction was concentrated to 0.1 ml under reduced pressure at room temperature, and the concentrate was subjected to thin-layer chromatography (TLC).

Silica Gel H was used according to Stahl (Kieselgel H nach Stahl, Art 7736, E. Merck, Darmstadt, Germany). Gel (30 g) was shaken with 70 ml of distilled water in a 150-ml stoppered flask for 60 s. This mixture was spread on glass sheets. Gel layers (20 by 20 cm; 0.25 mm thick) were prepared and dried for 30 min at 110 C in a hot-air oven. The gel layers were cleaned by an acetone run and activated at 120 C for 30 min. The samples were placed, with a micropipette, on the layer at intervals of 4 cm. The

TABLE 1. *List of strains*^a

Species/reference	Strain no.	Strain no. received	Source
<i>M. kansasii</i> Hauduroy 1955 (8)	07001 07002 07008	Forbes-84 Bostrum D35 P-18	K. Takeya; E.H. Runyon K. Takeya; E.H. Runyon K. Takeya; E.H. Runyon
<i>M. marinum</i> Aronson 1926 (1)	08002 08003 08010	B-916 B-913 ATCC 927	K. Konno; A.J. Ross K. Konno; A.J. Ross ATCC
<i>M. avium</i> Chester 1901 (6)	11016 11017 11020	ATCC 15769 ATCC 19075 ATCC 15773	ATCC ATCC ATCC
<i>M. intracellulare</i> Runyon 1967 (20)	13001 13082 13083	P-40 ATCC 19077 ATCC 19178	K. Takeya; E.H. Runyon ATCC ATCC
<i>M. nonchromogenicum</i> Tsukamura 1965 (25)	09003 09023 09033	ATCC 19530 ATCC 19532 ATCC 19533	ATCC; Tsukamura 317 ATCC; Tsukamura 1721 ATCC; Tsukamura 1731
<i>M. terrae</i> Wayne 1966 (34)	38001 38002 38013	W-167 W-168A ATCC 15755	L.G. Wayne L. G. Wayne ATCC; Wayne W-45
<i>M. novum</i> Tsukamura 1967 (27)	24018 24021 24022	ATCC 19619 ATCC 19621 ATCC 19622	ATCC; Tsukamura 1945 ATCC; Tsukamura 1952 ATCC; Tsukamura 1954
<i>M. triviale</i> Kubica 1970 (12)	37003 37004 37013	C1614 C5181 T254-3	G.P. Kubica G.P. Kubica G.P. Kubica
<i>M. marianum</i> (<i>M. scrofulaceum</i>) Suzanne and Penso 1953 (23)	12002 12107 12108	P-6 ATCC 15978 ATCC 19073	K. Takeya; E.H. Runyon ATCC ATCC
<i>M. gordonae</i> Bojalil et al. 1962 (3)	T-12109 T-12110 T-12177	ATCC 14470 ATCC 19277 ATCC 23283	ATCC ATCC ATCC
<i>M. phlei</i> Lehmann and Neumann 1899 (15)	14001 14002 14003 14004 14022	SN101 SN102 SN103 SN104 ATCC 19249	R. Bönicke R. Bönicke R. Bönicke R. Bönicke ATCC
<i>M. smegmatis</i> Lehmann & Neumann 1899 (15)	17001 17002 17003	SN1 SN2 SN3	R. Bönicke R. Bönicke R. Bönicke

^a R. Bönicke, Forschungsinstitut Borstel, 2061 Borstel, West Germany. G. P. Kubica, Trudeau Institute, Saranac Lake, N.Y., USA. K. Konno, Tohoku University, Sendai, Japan. K. Takeya, Kyushu University, Fukuoka, Japan. L. G. Wayne, Veterans Administration Hospital, Long Beach, Calif., USA. NCTC, National Collection of Type Cultures, Central Public Health Laboratory, London, England. ATCC, American Type Culture Collection, Rockville, Md., USA.

TABLE 1—Continued

Species/reference	Strain no.	Strain no. received	Source
	17004	SN4	R. Bönicke
	17027	ATCC 14468	ATCC
<i>M. vaccae</i> Bönicke and Juhasz 1964 (4)	21007	ATCC 15483	ATCC
	21008	ATCC 23002	ATCC
	21009	ATCC 23004	ATCC
	21010	ATCC 23005	ATCC
	21011	ATCC 23014	ATCC
<i>M. aurum</i> Tsukamura 1966 (26)	15001	NCTC 10439	NCTC; Tsukamura 303
	15003	Exp 327	M. Tsukamura
	15005	Exp 352	M. Tsukamura
	15006	ATCC 23366	ATCC; Tsukamura 358
	15011	NCTC 10438	NCTC; Tsukamura 312
<i>M. thermoresistibile</i> Tsukamura 1966 (26)	01002	ATCC 19528	ATCC; Tsukamura 1002
	01028	ATCC 19529	ATCC; Tsukamura 1028
	01029	exp. 1029	M. Tsukamura
	01030	exp. 1030	M. Tsukamura
	01039	ATCC 19527	ATCC; Tsukamura 316
<i>M. fortuitum</i> Da Costa Cruz 1938 (7)	18001	ATCC 19709	ATCC; Tsukamura 335
	18008	ATCC 6841	ATCC; J. Da Costa Cruz
	18009	NCTC 8697	NCTC
	18109	ATCC 6842	ATCC; J. Da Costa Cruz
	18110	ATCC 14467	ATCC
<i>M. chelonae</i> subsp. <i>chelonae</i> Bergey et al. 1923 (2)	19001	SN 281	R. Bönicke
	19002	SN 282	R. Bönicke
	19003	SN 283	R. Bönicke
	19009	ATCC 19235	ATCC; R. Bönicke
	19010	ATCC 19237	ATCC; R. Bönicke
<i>M. chelonae</i> subsp. <i>abscessus</i> Moore and Frerichs 1953 (19)	22011	ATCC 14472	ATCC
	22012	ATCC 19977	ATCC
	22013	ATCC 23006	ATCC
	22014	ATCC 23007	ATCC
	22015	ATCC 23016	ATCC
<i>M. chitae</i> Tsukamura 1967 (28)	31001	NCTC 10485	NCTC; M. Tsukamura
	31003	ATCC 19628	ATCC; M. Tsukamura
	31004	ATCC 19629	ATCC; M. Tsukamura
	31005	ATCC 19630	ATCC; M. Tsukamura
	31008	ATCC 19627	ATCC; M. Tsukamura
<i>M. flavescens</i> Bojalil et al. 1962 (3)	33001	ATCC 14474	ATCC
	33002	Expt 3362	M. Tsukamura
	33003	Expt 3363	M. Tsukamura
	33004	Expt 3364	M. Tsukamura
	33005	Expt 3365	M. Tsukamura

following developing solvent was used in 13- to 14-cm unidimensional runs: *n*-propanol-*n*-butanol-water-ammonia (57:20:20:3, vol/vol/vol/vol). Developed chromatograms were dried at 100 C for 30 min and then subjected to scanning of the radioactivity by an automatic TLC scanner (Nihon-Musen Co., Tokyo,

Japan) (slit, 6 by 30 mm; range, 1,000 counts/5 s; chart speed, 300 mm/h; recording speed, 150 mm/h).

RESULTS

Distribution of the radioactivity into the lipid fraction (ethyl ether-ethanol-soluble

fraction) and residue fraction. Among the slowly growing mycobacterial species tested, the radioactivity of the lipid fraction was significantly lower in *M. avium* and *M. intracellulare* than in other species (Table 2). Among the rapidly growing mycobacteria tested, *M. smegmatis* and *M. chelonae* showed the lowest radioactivity in their lipid fraction, *M. vaccae* and *M. chitae* showed the highest, and the others were intermediate. In contrast to the above, the radioactivity in the residue fraction did not differ significantly among various species and was much greater than that in the lipid fractions (Table 2). *M. avium* seemed, exceptionally, to show low radioactivity in the residue fraction also.

TLC of the ethyl ether-ethanol-soluble fraction (lipid fraction). The results of the TLC method of investigation are summarized in Table 3 and illustrated in Fig. 1-7. Thin-layer chromatograms of the lipid fractions obtained from cultures of *M. nonchromogenicum* strain 09003, one 7 days old, one 14 days old, and one 21 days old, and from cultures of *M. chitae* strain 31003, one 3 days old, one 7 days old, and one 14 days old, are shown in Fig. 1. The results suggest that the distribution pattern of radioactive spots is not greatly influenced by the cultivation period

of the test organisms but that the organisms obtained from old cultures tended to show lower activity. In view of these results, the cultivation periods of 14 and 5 days were used in slowly growing and rapidly growing mycobacteria, respectively. Strains belonging to the same species showed similar patterns. The results for *M. nonchromogenicum*, *M. avium*, and *M. thermoresistibile* are shown as examples in Fig. 2 and 3. All three strains of *M. avium* and all three strains of *M. thermoresistibile* showed the same pattern (Fig. 2 and 3). On the other hand, two strains of *M. nonchromogenicum* showed the same pattern, but one strain showed a slightly different pattern, lacking a spot (Fig. 2).

All three strains of *M. kansasii* and all three strains of *M. marinum* showed the same patterns, one of each of which is illustrated in Fig. 4. These two photochromogens were differentiated clearly by the pattern.

Strains of the species of group II seemed to be heterogenous as to pattern. Of two strains (12008 and 12107) of *M. marianum* (synonym *M. scrofulaceum*), the latter strain lacked two spots that the former showed (Fig. 4). Another strain (12002) had the same pattern as that of strain 12008. Three strains of *M. gordonae* showed slightly different patterns (Table 3).

TABLE 2. Distribution of the radioactivity into ethyl ether-ethanol-soluble and residue fractions

Species	No. of strains	Radioactivity (counts/min) ^a	
		Ethyl ether-ethanol-soluble fraction ($\times 30$)	Residue fraction ($\times 10$)
<i>M. kansasii</i>	3	402 $\pm$ 142	17,280 $\pm$ 3,272
<i>M. marinum</i>	3	874 $\pm$ 254	22,151 $\pm$ 7,329
<i>M. avium</i>	3	158 $\pm$ 63	9,637 $\pm$ 5,348
<i>M. intracellulare</i>	3	149 $\pm$ 23	17,606 $\pm$ 3,541
<i>M. nonchromogenicum</i>	3	1,379 $\pm$ 702	23,214 $\pm$ 6,338
<i>M. terrae</i>	3	366 $\pm$ 164	14,945 $\pm$ 2,863
<i>M. novum</i>	3	893 $\pm$ 625	23,539 $\pm$ 4,827
<i>M. triviale</i>	3	299 $\pm$ 160	23,613 $\pm$ 1,762
<i>M. marianum</i> (<i>M. scrofulaceum</i>)	3	512 $\pm$ 25	15,972 $\pm$ 1,768
<i>M. gordonae</i>	3	640 $\pm$ 601	27,285 $\pm$ 1,109
<i>M. phlei</i>	5	1,077 $\pm$ 521	26,510 $\pm$ 2,221
<i>M. smegmatis</i>	5	540 $\pm$ 179	30,506 $\pm$ 1,280
<i>M. vaccae</i>	5	3,850 $\pm$ 1,199	28,370 $\pm$ 4,387
<i>M. aurum</i>	5	1,880 $\pm$ 698	32,621 $\pm$ 4,705
<i>M. thermoresistibile</i>	5	1,930 $\pm$ 1,211	21,980 $\pm$ 4,654
<i>M. fortuitum</i>	5	1,087 $\pm$ 523	34,552 $\pm$ 2,798
<i>M. chelonae</i> subsp. <i>chelonae</i>	5	426 $\pm$ 151	25,109 $\pm$ 4,315
<i>M. chelonae</i> subsp. <i>abscessus</i>	5	696 $\pm$ 273	24,500 $\pm$ 6,172
<i>M. chitae</i>	5	3,768 $\pm$ 1,416	31,822 $\pm$ 1,168
<i>M. flavescens</i>	5	968 $\pm$ 415	29,718 $\pm$ 2,618

^a (Mean in three or five strains) $\pm$ (standard deviation). The radioactivity shows the activity measured in a 0.2-ml sample. Since the amounts of ethyl ether-ethanol-soluble and residue fractions were 6.0 and 2.0 ml, respectively, the total radioactivity in the fraction can be obtained by multiplying the counts per minute by 30 and 10, respectively.

TABLE 3. Radioactive spots in thin-layer chromatograms of ethyl ether-ethanol-soluble fraction (lipid fraction)^a

Species	Radioactive spots				
	a	b	c	d	e
<i>M. kansasii</i>	a	b	c		E
<i>M. marinum</i>	a	b	c		
<i>M. avium</i>					E
<i>M. intracellulare</i>					E*
<i>M. nonchromogenicum</i>		b	c	d*	E
<i>M. terrae</i>	a	b	c		
<i>M. novum</i>	a*	b	c	d*	
<i>M. triviale</i>	a	b	c		
<i>M. marianum</i> (<i>M. scrofulaceum</i>)	a	b*	c*		
<i>M. gordonae</i>	a	b	c*		e*
<i>M. phlei</i>	A*	B	c		e
<i>M. smegmatis</i>	a				E
<i>M. vaccae</i>		B	c*		
<i>M. aurum</i>		b*	c*		
<i>M. thermoresistibile</i>			C	d	
<i>M. chitae</i>		b	C	d	
<i>M. fortuitum</i>	a				
<i>M. chelonae</i> subsp. <i>chelonae</i>	a	b*			E
<i>M. chelonae</i> subsp. <i>abscessus</i>	a				E
<i>M. flavescens</i>	a	b*	C*		e

^a Spot a, R_f 0.14 to 0.15; spot b, R_f 0.25 to 0.27; spot c, R_f 0.39 to 0.43; spot d, R_f 0.56 to 0.59; spot e, R_f 0.98 to 1.00. Spots indicated with a capital letter were the most prominently radioactive; for those indicated with an asterisk, the spots may be absent in one of three or in one or two of five strains tested. *M. intracellulare* strains 13001 and 13083 lack spot e and show no marked spot. *M. nonchromogenicum* strain 09003 lacks spot d. *M. novum* strain 24021 lacks spots a and d. *M. marianum* (*M. scrofulaceum*) strain 12107 lacks spots b and c. *M. gordonae* strain T-12177 shows a, b, c and e; strain T-12109 lacks spot c; and strain T-12110 lacks spot e and shows a small spot a. *M. phlei* strain 14001 lacks spot a. *M. vaccae* strain 21011 lacks spot c. *M. aurum* strain 15001 lacks spots b, and strain 15006 lacks spot c. *M. fortuitum* strains 18001 and 18109 show spots b and c. *M. chelonae* subsp. *chelonae* strains 19003 and 19010 lack spot b. *M. chelonae* subsp. *abscessus* strain 22014 shows spot b. *M. flavescens* strain 33001 lacks spots b and c.

Strain T-12109 showed three spots, a, b, and e (Fig. 5A); strain T-12110 showed three spots, a, b, and c (Fig. 5C); and strain T-12177 showed four spots, a, b, c, and e. The results suggest that *M. gordonae* may be more heterogeneous than other species.

Strains of *M. intracellulare* showed the same pattern (Fig. 5A). (It was very similar to that of *M. avium* [Fig. 2].) All three strains of *M. terrae* (Fig. 5E) and three strains of *M. triviale* (Fig. 5F) showed the same pattern, suggesting that these two species may be identical. Strains of *M. novum* (Fig. 5D) showed a pattern slightly different from that of *M. terrae* and *M. triviale*. Among nonpathogenic group III mycobacteria, the pattern for *M. nonchromogenicum* (Fig. 2A, B, C) could be distinguished from those of the

other three, *M. novum*, *M. terrae* and *M. triviale* (Fig. 5D, E, F). However, the patterns of these four species showed some similarity. The patterns of rapidly growing mycobacteria are shown in Fig. 6 and 7. Usually, strains belonging to the same species showed the same pattern. However, three strains of *M. aurum* showed two spots and two strains showed only one.

M. thermoresistibile and *M. chitae* showed similar patterns, although these two species differ distinctly from each other in their phenetic characters (29). Four strains of *M. flavescens* showed four spots, whereas one strain showed two spots. Two subspecies of *M. chelonae* differed slightly in their patterns (Fig. 6).

DISCUSSION

The purpose of this study was to investigate the possibility of using TLC of the lipid fraction for differentiation among mycobacterial species. The ethyl ether-ethanol extraction method was chosen to simplify the procedure, although it might be insufficient for extraction of all lipids. This method gives results which may be useful for differentiation among certain mycobacterial species. We do not propose to investigate the method in greater depth. The information may be of use to others for further research, the possibilities of which are illustrated by the following. Both *M. kansasii* and *M. marinum* showed three common spots, but the former was differentiated from the latter by the presence of a fourth prominent spot (Fig. 4; Table 3). *M. avium* and *M. intracellulare* showed the same pattern (Fig. 2; Fig. 5). These two were considered to belong to the same species by the IWGMT (18).

In an IWGMT study, *M. nonchromogenicum* and *M. terrae* were considered to be closely related but of two different species (18). The results of our study suggest that these are differentiable by their patterns, although the patterns are of considerable similarity. Although *M. novum* was considered to be a synonym of *M. terrae* in an IWGMT study (18), these two showed slightly different patterns (Fig. 5). *M. terrae* and *M. triviale* showed the same pattern (Fig. 5). *M. marianum* (*M. scrofulaceum*) and *M. gordonae* appear relatively heterogeneous with respect to their patterns, but these were distinguishable from the others.

Among rapidly growing mycobacteria, *M. phlei* and *M. smegmatis* showed characteristic patterns (Fig. 6). The patterns of *M. chitae* and *M. thermoresistibile* were similar to each other but different from those of the others (Fig. 6

FIG. 1. Radioactive spots in thin-layer chromatogram of ethyl ether-ethanol-soluble fraction of *M. nonchromogenicum* (strain 09003) and *M. chitae* (strain 31003). (A) *M. nonchromogenicum* 09003 (a 21-day-old culture). (B) *M. nonchromogenicum* 09003 (a 14-day-old culture). (C) *M. nonchromogenicum* 09003 (a 7-day-old culture). (D) *M. chitae* 31003 (a 14-day-old culture). (E) *M. chitae* 31003 (a 7-day-old culture). (F) *M. chitae* 31003 (a 3-day-old culture). Cultures of various ages (days) were incubated with [35 S]methionine for 20 h, and their ethyl ether-ethanol-soluble fractions were subjected to TLC.

FIG. 2. Radioactive spots in thin-layer chromatogram of ethyl ether-ethanol-soluble fraction of three strains of *M. nonchromogenicum* and three strains of *M. avium*. (A) *M. nonchromogenicum* 09033. (B) *M. nonchromogenicum* 09023. (C) *M. nonchromogenicum* 09003. (D) *M. avium* 11017. (E) *M. avium* 11016. (F) *M. avium* strain 11020.

FIG. 3. Radioactive spots in thin-layer chromatogram of ethyl ether-ethanol-soluble fraction of three strains of *M. thermoresistible*. (A) *M. thermoresistible* 01002. (B) *M. thermoresistible* 01030. (C) *M. thermoresistible* 01028.

FIG. 4. Radioactive spots in thin-layer chromatogram of ethyl ether-ethanol-soluble fraction of *M. kansasii*, *M. marinum*, and *M. marianum* (*M. scrofulaceum*). (A) *M. marinum* 08010. (B) *M. kansasii* 07001. (C) *M. marianum* (*M. scrofulaceum*) 12107. (D) *M. marianum* (*M. scrofulaceum*) 12108.

FIG. 5. Radioactive spots in thin-layer chromatogram of ethyl ether-ethanol-soluble fraction of *M. intracellulare*, *M. gordonae*, *M. novum*, *M. terrae*, and *M. triviale*. (A) *M. intracellulare* 13082. (B) *M. gordonae* T-12109. (C) *M. gordonae* T-12110. (D) *M. novum* 24018. (E) *M. terrae* 38013. (F) *M. triviale* 37003.

FIG. 6. Radioactive spots in thin-layer chromatogram of ethyl ether-ethanol-soluble fraction of *M. fortuitum*, *M. chelonae* subsp. *chelonae*, *M. chelonae* subsp. *abscessus*, *M. smegmatis*, *M. phlei*, and *M. thermoresistibile*. (A) *M. fortuitum* 18104. (B) *M. chelonae* subsp. *chelonae* 19002. (C) *M. chelonae* subsp. *abscessus* 22015. (D) *M. smegmatis* 17027. (E) *M. phlei* 14022. (F) *M. thermoresistibile* 01039.

FIG. 7. Radioactive spots in thin-layer chromatogram of ethyl ether-ethanol-soluble fraction of *M. vaccae*, *M. aurum*, *M. flavescens*, and *M. chitae*. (A) *M. vaccae* 21009. (B) *M. aurum* 15001. (C) *M. aurum* 15006. (D) *M. flavescens* 33004. (E) *M. flavescens* 33001. (F) *M. chitae* 31003.

and 7). Two subspecies of *M. chelonae* showed slightly different patterns (Fig. 6). *M. vaccae* and *M. aurum* showed similar patterns (Fig. 7), but the type strain of *M. aurum* (15006; ATCC 23366) lacked one spot.

Strains identified as *M. flavescens* seemed to be heterogenous with respect to the pattern (Fig. 7). Four strains isolated in Japan (Table 1) had once been identified as *M. gallinarum* (32) and later were identified as *M. flavescens* (Tsukamura, personal communication to the IWGMT, 1973). These strains had a different pattern from that of *M. flavescens* (type strain ATCC 14474). Phenetically, there are few differences between *M. flavescens* and *M. gallinarum* (30, 32).

REPRINT REQUESTS

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