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Application of Colorimetric Microdilution Plate Hybridization for Rapid Genetic Identification of 22 *Mycobacterium* Species

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Quantitative microdilution plate hybridization was used to identify 22 *Mycobacterium* species. DNAs of clinical strains were rapidly extracted and labeled with photoreactive biotin. Labeled DNAs were distributed into wells of a microdilution plate in which reference DNAs had been immobilized. After 2 h of hybridization, hybridized DNAs were quantitatively detected with peroxidase-conjugated streptavidin and the substrate, tetramethylbenzidine. This method could differentiate among 20 of the 22 *Mycobacterium* species tested. The type strains of *Mycobacterium tuberculosis* and *M. bovis* were genetically highly related and could not be differentiated by this method. Of 194 biochemically identified human clinical strains, 178 (90%) were genetically identified within 3 h of the small-scale DNA extraction.

Identifying *Mycobacterium* species by conventional methods requires at least 1 month because the bacterium grows slowly on solid media. A method for rapidly identifying mycobacteria would be a significant advantage for clinicians who would like to begin chemotherapies for their patients as soon as possible.

The genetic identification technique is a new approach which shortens the time required for mycobacterial identification. Several specific DNA probes have been developed for the identification and detection of the *Mycobacterium tuberculosis* group, *M. intracellulare*, and *M. avium* (1, 15, 16). However, these specific DNA probes have been designed only to identify specific species. For more than 20 remaining pathogenic species, such probes are not available.

We have recently developed a rapid method of genetic identification for bacteria that does not use any specific DNA probes but instead uses total chromosomal DNA labeled with nonradioactive photoreactive biotin (2, 3). This method uses a technique for quantifying the genetic relatedness among bacterial strains with a DNA-DNA hybridization method which has previously been used for bacterial taxonomy (10, 22). Using this method, we established genetic identification methods for legionellae, nonfermenters, anaerobic rods, and gram-positive cocci (2, 4, 5) that required only 3 to 4 h.

However, the application of the method for identifying *Mycobacterium* species necessitated many modifications. Because the mycobacteria resisted the standard lysing protocols designed for gram-positive cocci and gram-negative rods, we developed a small-scale lysing procedure for the mycobacteria and established a quick DNA labeling method. This report describes our 4-h genetic identification method for 22 species of the genus *Mycobacterium*.

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MATERIALS AND METHODS

Bacterial strains. Reference strains from which DNA was extracted and immobilized in microdilution plates are listed in Table 1. Twenty-two type strains, 1 reference strain (ATCC 14467, labeled as "*M. peregrinum*"), 1 clinical strain (biochemically identified as *M. gordonae* KPM 2205), and *Escherichia coli* ATCC 25922 were used. Two hundred nine human clinical strains were used for evaluating the microdilution plate hybridization.

Preparation of reference DNA. Rapid growers were cultured on 1% Ogawa medium for 7 days, and slow growers were cultured on 1% Ogawa medium for 3 weeks. Cells were harvested, suspended in 10 mM Tris-EDTA buffer (pH 8.0), and centrifuged at 10,000 × *g* for 10 min. Sedimented cells were resuspended in Tris-EDTA buffer, and lysozyme was added to each cell suspension (final concentration, 1 mg/ml). The suspensions were incubated at 37°C. After 18 h of incubation, sodium dodecyl sulfate was added to the suspensions (final concentration, 1%), which were further incubated at 60°C for 1 h. After this incubation, the suspensions were centrifuged at 10,000 × *g* for 10 min and sedimented cells were suspended in TE buffer and treated with an ultrasonic sonicator (model UP 200P; Tomy Seiko Co., Tokyo, Japan) for 5 min. The centrifuged supernatant fluid and sonicated extract were combined and digested with proteinase K (final concentration, 200 μg/ml) at 37°C for 1 h. An equal volume of phenol-chloroform-isoamyl alchohol (25:24:1) was added to the suspensions, which were then shaken for 20 min to remove the protein. Following ethanol precipitation, RNase treatment was performed by a previously described method to purify the DNA (5).

Immobilization of reference DNA in wells of microdilution plates. Reference DNA was immobilized in wells of microdilution plates by a previously described method (3), with minor modifications. In brief, 100 μl of unlabeled single-stranded reference DNA solution (5 μg/ml in phosphate-buffered saline containing 0.1 M $MgCl_2$) was distributed into wells of microdilution plates (Immunoplate; Nunc). The plates were kept at room temperature for 16 h, and the

TABLE 1. Reference strains immobilized for the microdilution plate assay

Organism	Strain	Status
M. tuberculosis	ATCC 27294	Type
M. bovis	ATCC 19210	Type
M. kansasii	ATCC 12478	Type
M. marinum	ATCC 927	Type
M. simiae	ATCC 25275	Type
M. scrofulaceum	ATCC 19981	Type
M. gordonae	ATCC 14470	Type
M. gordonae[a]	KPM 2205	Clinical isolate
M. szulgai	NCTC 10831	Type
M. avium	ATCC 25291	Type
M. intracellulare	ATCC 13950	Type
M. gastri	ATCC 15754	Type
M. xenopi	NCTC 10042	Type
M. nonchromogenicum	ATCC 19530	Type
M. terrae	ATCC 15755	Type
M. triviale	ATCC 23292	Type
M. fortuitum	ATCC 6841	Type
"*M. peregrinum*"	ATCC 14467	
M. chelonae subsp. *chelonae*	NCTC 946	Type
M. chelonae subsp. *abscessus*	ATCC 19977	Type
M. chitae	ATCC 19627	Type
M. phlei	ATCC 11758	Type
M. flavescens	ATCC 14474	Type
M. vaccae	ATCC 15483	Type
E. coli	ATCC 25922	Strain B

[a] *M. gordonae* KPM 2205 is genetically different from the type strain.

solution was replaced with 300 μl of phosphate-buffered saline containing 0.1 M $MgCl_2$ and 50 μg of denatured salmon DNA per ml. After 2 h of incubation, the solution was discarded and the plates were dried at 50°C for 2 h. The plates were kept in a dry condition.

Biochemical identification of mycobacterial strains. Human clinical strains were tentatively identified by 12 characteristics (see Table 3).

Preparation of labeled DNA from human clinical strains. One-quarter to one-half loopful of mycobacterial cells (approximately 3 mm^3) was harvested and transferred to a test tube (diameter, 16 mm) filled with 2 g of glass beads (diameter, 2 mm) and 50 μl of 10% Tween 80. The test tube was vortexed for 30 s, and 1 ml of 0.25 M NaCl–1 mM EDTA (pH 8.0) was added. One milliliter of phenol-chloroform-isoamyl alchohol (25:24:1) was added, and the test tube was vortexed for another 30 s. The test tube was centrifuged at 3,000 × *g* for 5 min, and 500 μl of supernatant fluid was transferred to an Eppendorf tube. One milliliter of ethanol was added to the tube. After the tube contents were mixed, DNA was precipitated by centrifugation at 16,000 × *g* for 5 min. The ethanol was discarded, and 100 μl of photobiotin solution (40 μg/ml) was added to the tube and irradiated by a 500-W lamp as described previously (3, 5). At this point, 200 μl of *n*-butanol was added to the mixture to remove excess photobiotin. After the *n*-butanol layer was removed, the labeled DNA was denatured by the addition of 50 μl of 0.6 N NaOH. The solution was neutralized by the addition of 50 μl of 0.9 M NaH_2PO_4.

Hybridization. Labeled single-stranded DNA was mixed with 3 ml of hybridization solution containing 50% formamide (3), and 100 μl of the solution was distributed into each microdilution plate well, in which the reference DNA had been immobilized. After hybridization at 60°C for 2 h, the wells were washed with saline-sodium citrate solution (3) three times. Streptavidin-horseradish peroxidase (100 μl; Zymed Laboratories Inc., San Francisco, Calif.) diluted 5,000 times with PBS was distributed into each well. After 10 min of incubation at 37°C, the wells were washed three times with 1× SSC (0.15 M NaCl plus 0.015 M sodium citrate) and filled with 100 μl of substrate solution (50 μl of tetramethylbenzidine [400 μg/ml] and 50 μl of H_2O_2 [30 μg/ml]). The plates were incubated at room temperature, and the optical density at 630 nm (OD_{630}) of each well was measured within 30 min.

Genetic identification of clinical strains. DNAs from clinical strains (see Table 3) were labeled with photobiotin and hybridized to the reference DNAs immobilized in the microdilution plate wells. The extent of hybridization was determined by colorimetric measurement. The OD_{630} of the highest color intensity reached 0.5 usually within 30 min after the addition of substrates. When the ratio of the maximum color intensity and the color intensity of the negative control well (value for *E. coli*) (maximum value/negative value) was higher than 1.9, the data were processed as follows. The value of the maximum color intensity was taken to be 100%, and that of the control well, in which the DNA from *E. coli* was immobilized, was taken to be 0%. When the relative relatedness of the well which emitted the second strongest color intensity was lower than 70%, the labeled strain was considered to be identified.

RESULTS

Reidentification of reference strains by microdilution plate hybridization. The method was evaluated by reidentifying reference strains (Table 2). The type strains for *M. tuberculosis* and *M. bovis* could not be differentiated by this method; however, 20 other species could clearly be recognized. The relative similarity between *M. tuberculosis* and *M. bovis* under stringent conditions (hybridization at 60°C in 2× SSC–50% formamide) was between 95 and 100%.

The name "*M. peregrinum*" does not appear in the *Approved Lists of Bacterial Names* (18), and this species is considered to belong to the *M. fortuitum* group. However, strain ATCC 14467, labeled as "*M. peregrinum*," was genetically different from the type strain of *M. fortuitum*. *M. chelonae* is divided into two subspecies, *M. chelonae* subsp. *chelonae* and *M. chelonae* subsp. *abscessus* (11); however, their relatedness was less than 30% under our experimental conditions.

Identification of clinical strains by microdilution plate hybridization. When our microdilution plate hybridization test was applied to the identification of clinical strains, DNAs of clinical strains were not immobilized in microdilution plates. Thus, an absolute homology value between a clinical strain and a type strain could not be calculated. We therefore used two criteria for the identification of clinical strains. The first criterion is that the maximum value/negative value should be higher than 1.9, because the values were always higher than 1.9 when reference strains were reidentified. Second, since homology values among established species should be lower than 70%, according to the recommendation of the ad hoc committee (22), the value for the well showing the second highest reaction should be always less than 70% of the maximum value obtained.

During our study of clinical strains, we noted a few strains in which the second criterion but not the first criterion was satisfied. We repeated the experiments for these strains and finally identified them as one of the established species used in this study. We believe that the DNAs were not properly

TABLE 2. Reidentification of 24 reference strains by microdilution plate hybridization

Immobilized organism	Relative color intensity[a] with the following test organism:																							
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1 *M. tuberculosis*	100	95	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
2 *M. bovis*	100	100	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
3 *M. kansasii*	—	—	100	—	—	—	—	—	—	—	—	54	—	—	—	—	32	—	—	36	—	—	—	—
4 *M. marinum*	—	—	—	100	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
5 *M. simiae*	—	—	—	—	100	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
6 *M. scrofulaceum*	—	—	—	—	—	100	—	—	—	—	30	—	—	—	—	—	—	—	—	—	—	—	—	—
7 *M. gordonae*[b]	—	—	—	—	—	—	100	37	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
8 *M. gordonae*[c]	—	—	—	—	—	—	37	100	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
9 *M. szulgai*	—	—	—	—	—	—	—	—	100	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
10 *M. avium*	30	—	—	—	31	49	—	—	—	100	53	—	—	—	—	—	—	—	31	41	—	—	—	37
11 *M. intracellulare*	—	—	—	—	—	38	—	—	—	44	100	—	—	—	—	—	—	—	—	—	—	—	—	—
12 *M. gastri*	—	—	61	—	—	—	—	—	—	—	—	100	—	—	—	—	—	—	—	—	—	—	—	—
13 *M. xenopi*	—	—	—	—	—	—	—	—	—	—	—	—	100	—	—	—	—	—	—	—	—	—	—	—
14 *M. nonchromogenicum*	—	—	—	—	—	—	—	—	—	—	—	—	—	100	42	—	—	—	—	—	—	—	—	—
15 *M. terrae*	—	—	—	—	—	—	—	—	—	—	—	—	—	—	100	—	—	—	—	—	—	—	—	—
16 *M. triviale*	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	100	30	—	—	—	—	—	—	—
17 *M. fortuitum*	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	100	49	—	—	—	—	—	—
18 "*M. peregrinum*"	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	49	100	—	—	—	—	—	—
19 *M. chelonae* subsp. *chelonae*	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	100	—	—	—	—	—
20 *M. chelonae* subsp. *abscessus*	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	100	—	—	—	—
21 *M. chitae*	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	100	—	—	—
22 *M. phlei*	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	100	—	30
23 *M. flavescens*	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	100	36
24 *M. vaccae*	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	40	—	—	—	100
OD_{630} of control well[d]	0.08	0.07	0.07	0.10	0.07	0.12	0.10	0.10	0.06	0.06	0.08	0.08	0.08	0.08	0.09	0.08	0.07	0.07	0.07	0.13	0.08	0.07	0.07	0.12
OD_{630} of maximum well	0.31	0.34	0.24	0.22	0.35	0.34	0.25	0.69	0.21	0.25	0.37	0.26	0.34	0.37	0.39	0.36	0.31	0.42	0.32	0.25	0.37	0.30	0.26	0.37
Maximum/negative ratio	3.88	4.86	3.43	2.20	5.00	2.83	2.50	6.90	3.50	4.17	4.63	3.25	4.25	4.63	4.33	4.50	4.43	6.00	4.57	1.92	4.63	4.29	3.71	3.08

[a] —, color intensity below 30%.
[b] ATCC 14470 (type strain).
[c] KPM 2205 (clinical isolate).
[d] Ten minutes after the addition of substrates.

TABLE 3. Selected characteristics for tentative identification of mycobacteria isolated from human clinical specimens[a]

Runyon's group	Species[b]	Growth at 37°C within 7 days	Colony[c]	Scotochromogenic	Photochromogenic	PAS hydrolysis	Picrate (0.20%)	Arylsulfatase	Tween 80 hydrolysis	Urease	Nitrate reduction	Niacin	Semiquantitative catalase
M. tuberculosis group	*M. tuberculosis*	−	R	−	−	−	−		−	+	+	+	−
	M. bovis	−	S (R)	−	−	−	−		−	+	−	−	−
I	*M. kansasii*	−	R, S	−	Yellow	−	−		+	+	+	−	+
	M. marinum	−	S	−	Yellow	−	−		+	+	−	−	∓
	M. simiae	−	S	−	Yellow	−	−		−	+	−	+	+
II	*M. scrofulaceum*	−	S	Orange	Orange	−	−		−	±	−	−	+
	M. szulgai	−	S	Orange	Orange	−	−		−[d]	+	+	−	+
	M. gordonae	−	S	Orange	Orange	−	−		+	∓	−	−	+
III	*M. avium* complex	−	S	−	−	−	−		−	−	−	−	−
	M. xenopi	−	S	Yellow or −	Yellow or −	−	−		−	−	−	−	−
	M. gastri	−	S	−	−	−	−		+	+	−	−	−
	M. nonchromogenicum complex	−	S, R	−	−	−	−		+	−	V	−	+
IV	*M. fortuitum*	+	S (R)	−	−	+	+	+	+	+	+	−	
	M. chelonae subsp. *chelonae*	+	S (R)	−	−	+	−	+	−	+	−	−	
	M. chelonae subsp. *abscessus*	+	S (R)	−	−	+	+	+	−	+	−	−	
	Other	+	S (R)	Yellow or − Orange	Yellow or − Orange	−	+	−	+	+	+	−	

[a] Data were taken from references 9 and 17. +, more than 90% of strains positive; ±, 70 to 90% of strains positive; V, 31 to 69% of strains positive; ∓, 10 to 30% of strains positive; −, less than 10% of strains positive.

[b] *M. avium* complex is *M. avium* and *M. intracellulare*; *M. nonchromogenicum* complex is *M. nonchromogenicum*, *M. terrae*, and *M. triviale*.

[c] R, rough; S, smooth; (R), a few strains rough.

[d] 51 to 84% of strains positive in reference 23.

TABLE 4. Identification of 209 human clinical strains by microdilution plate hybridization

Tentative name[a] (*n*)	Genetic identification (*n*)
M. tuberculosis (30)	*M. tuberculosis* (30)
M. kansasii (20)	*M. kansasii* (20)
M. marinum (2)	*M. marinum* (2)
M. simiae (2)	*M. simiae* (2)
M. scrofulaceum (26)	*M. scrofulaceum* (14), *M. avium* (3), *M. gordonae* (6), unidentified (3)
M. gordonae (15)	*M. gordonae* (12)[b], *M. gordonae* (2)[c], *M. avium* (1)
M. szulgai (2)	*M. szulgai* (2)
M. avium complex (30)	*M. avium* (17), *M. intracellulare* (10), unidentified (3)
M. gastri (2)	*M. gastri* (2)
M. xenopi (3)	*M. xenopi* (3)
M. nonchromogenicum complex (20)	*M. terrae* (8), *M. triviale* (2), *M. nonchromogenicum* (4), unidentified (6)[d]
M. fortuitum (30)	*M. fortuitum* (16), "*M. peregrinum*" (8), *M. chelonae* subsp. *chelonae* (3), *M. chelonae* subsp. *abscessus* (1), unidentified (2)
M. chelonae subsp. *chelonae* (10)	*M. chelonae* subsp. *chelonae* (7), *M. chelonae* subsp. *abscessus* (1), unidentified (2)
M. chelonae subsp. *abscessus* (2)	*M. chelonae* subsp. *abscessus* (2)
Unidentified (15)	Unidentified (15)

[a] Tentative names of clinical strains were determined by the 12 characteristics listed in Table 3.
[b] ATCC 14470.
[c] KPM 2205.
[d] These strains did not hybridize to the type strains of *M. shimoidei* (ATCC 27962) and *M. malmoense* (ATCC 29571).

extracted or labeled in such cases. Therefore, we expect that in some cases, an isolate might be misjudged as being false-negative for hybridization when only the first criterion is used. On the other hand, we expect that a strain which is an independent species but which is highly related to one of the established reference species by 60 to 69% homology might occasionally be misidentified. Therefore, one must be careful to use both of these two criteria for taxonomic purposes.

In this experiment, 194 biochemically identified strains and 15 unidentified strains were evaluated by microdilution plate hybridization. Of the 194 tentatively identified strains, 178 (90%) satisfied the above-described criteria. The remaining, slowly glowing 16 strains did not satisfy the above-described criteria. Strains which belonged to Runyon group IV but which could not be identified biochemically did not hybridize to any of the established 22 species used in this study.

In our preliminary study, we found that the 14 biochemically identified strains of *M. gordonae* contained genetically different organisms. It was not possible to biochemically differentiate them from the type strain with the 12 tests listed in Table 3; thus, we arbitrarily selected a clinical strain, KPM 2205, and immobilized its DNA in the microdilution plate. Two strains of *M. gordonae* hybridized to strain KPM 2205. *M. fortuitum* also contained genetically different organisms. Of the 30 biochemically identified strains, 8 did not hybridize to the type strain; instead, they hybridized to "*M. peregrinum*" ATCC 14467, an organism which is thought to belong to the *M. fortuitum* group (11). Two additional strains failed to hybridize to either *M. fortuitum* ATCC 6841 or "*M. peregrinum*" ATCC 14467 and are thus genetically independent.

We selected the group name, *M. nonchromogenicum* complex, for *M. nonchromogenicum*, *M. terrae*, and *M. triviale*, because the 12 biochemical tests were unable to differentiate among these three species. Of the 20 strains biochemically identified as *M. nonchromogenicum* complex, 14 were identified as *M. nonchromogenicum*, *M. terrae*, or *M. triviale*, as shown in Table 4. However, 6 strains did not hybridize to any of the three type strains.

DISCUSSION

In our previous study, we reported on quantitative fluorometric hybridization, a highly quantitative method. However, since the colorimetric method is less expensive and most clinical laboratories have already installed a microdilution plate spectrophotometer for various enzyme-linked immunosorbent assays, we used a colorimetric detection system with streptavidin-peroxidase and tetramethylbenzidine in this study. With this method, the reaction is not stopped after the addition of substrates; thus, the color intensity of each well of the microdilution plate continues to increase with time. The quantitative range of OD_{630} is very narrow when peroxidase and tetramethylbenzidine are used; therefore, we only used values lower than 0.8. In most cases, the well which developed the maximum blue color was obvious before the optical density was measured.

Modern taxonomic guidelines suggest that homology among individual independent species should be less than 70% under optimal conditions and that the ΔT_m should be less than 5°C (22). These criteria were met for most members of the genus *Mycobacterium*. However, *M. tuberculosis* and *M. bovis* did not satisfy these criteria. Their relatedness ranged from 95 to 100%. It has been previously reported that their homology, estimated by quantitative DNA hybridization, is above 90% (7, 8). Our data supported these previous findings. Thus, the difference between the two species is probably an intraspecies difference. They should be reclassified as a single species and differentiated as pathovars or biovars.

In practice, however, microbiologists may be required to differentiate the two species and can do so by simple tests such as niacin and thiophene-2-carboxylic acid hydrazide sensitivity. Imaeda (7) reported that *M. microti* and *M. africanum* are also genetically highly related to *M. tuberculosis* (we recently confirmed this fact, although the data are not included in this paper).

Separation of *M. intracellulare* from *M. avium* is often difficult by biochemical characterization (11, 23). The biochemical tests listed in Table 3 cannot differentiate between the two organisms. They are identified simply as *M. avium*

TABLE 5. Atypical characteristics of 11 misidentified strains[a]

Strain	Biochemical misidentification	Genetic identification	Growth at 37°C within 7 days	Colony[b]	Scoto-chromogenic	Photo-chromogenic	PAS hydrolysis	Picrate (0.20%)	Arylsulfatase	Tween 80 hydro-lysis[c]	Urease	Nitrate reduc-tion
KPM 2009	*M. scrofulaceum*	*M. gordonae*	−	S	Yellow	Yellow	−	−	−	−	−	−
KPM 2016	*M. scrofulaceum*	*M. gordonae*	−	S	Yellow	Yellow	−	−	−	−	−	−
KY 488	*M. scrofulaceum*	*M. gordonae*	−	S	Yellow	Yellow	−	−	−	−	−	−
KPM1988-5	*M. scrofulaceum*	*M. gordonae*	−	S	Yellow	Yellow	−	−	−	−	+	W
KPM 2013	*M. scrofulaceum*	*M. gordonae*	−	S	Yellow	Yellow	−	−	−	−	−	−
KPM 2014	*M. scrofulaceum*	*M. gordonae*	−	S	Yellow	Yellow	−	−	−	−	−	−
KY 370	*M. fortuitum*	*M. chelonae* subsp. *abscessus*	+	R, S	−	−	+	+	+	−	+	+
KY 659	*M. fortuitum*	*M. chelonae* subsp. *abscessus*	+	S	−	−	+	+	+	−	+	+
KY 660	*M. fortuitum*	*M. chelonae* subsp. *abscessus*	+	S	−	−	+	+	+	−	+	+
KY 734	*M. fortuitum*	*M. chelonae* subsp. *abscessus*	+	S	−	−	+	+	+	−	−	+
KY 750	*M. chelonae* subsp. *abscessus*	*M. chelonae* subsp. *chelonae*	+	R, S	−	−	+	+	+	−	+	−

[a] +, positive; −, negative; W, weakly positive. Boldfacing indicates atypical traits.
[b] S, smooth; R, rough.
[c] The test was repeated at least three times.

complex. However, 27 of 30 strains identified as *M. avium* complex were clearly separated into two species, *M. avium* and *M. intracellulare*, by hybridization (Table 4). *M. paratuberculosis*, an important animal pathogen, was not included in this study. Several recent reports suggest that this organism is genetically closely related to *M. avium* (6, 19, 21). The 30 strains identified as *M. avium* complex, however, did not require mycobactin for growth and thus were different from *M. paratuberculosis* (21). If a strain of *M. paratuberculosis* were present in a human clinical specimen, our hybridization system might fail to differentiate it from *M. avium*.

The group name *M. nonchromogenicum* complex was assigned to the three organisms *M. nonchromogenicum*, *M. terrae*, and *M. triviale* because they cannot be differentiated by the biochemical tests listed in Table 3. Fourteen strains were genetically identified as one of the three established species, but six strains did not hybridize with the DNA of any of the three type strains. We suspected that *M. malmoense* and *M. shimoidei* might have been identified as a member of the *M. nonchromogenicum* complex by the biochemical tests. Thus, we prepared two type strains (*M. shimoidei* ATCC 27962 and *M. malmoense* ATCC 29571) and carried out hybridization experiments between these two type strains and six unidentified strains. However, these six strains did not satisfy our identification criteria (data not shown); thus, we concluded that they were different from the two type strains.

In our preliminary study, we noted that strains identified as *M. gordonae* and *M. fortuitum* were divided into two genospecies. *M. gordonae* KPM 2205, which was isolated from a human patient, was genetically different from the type strain. We could not find biochemical characteristics to differentiate it from the type strain. We also confirmed that strain KPM 2205 was genetically different from the type strain of *M. asiaticum*, although *M. asiaticum* is also a chromogenic organism (data not shown). Eight strains biochemically identified as *M. fortuitum* did not hybridize to the type strain; instead, they hybridized to "*M. peregrinum*." An international cooperative study proposed that "*M. peregrinum*" should be a synonym for *M. fortuitum* (11). Furthermore, Minnikin et al. (13, 14) reported their relatedness from a study of their mycolic acid profiles. However, Levy-Frebault et al. (12) and Tsang et al. (20) suggested that "*M. peregrinum*" is genetically and antigenically independent. Our data support their observations. Differential characteristics which separate "*M. peregrinum*" from *M. fortuitum* should be selected for identification in clinical laboratories.

In Table 5, we prepared a list of biochemically atypical strains, all from our culture collection at the Kobayashi Pharmaceutical Co. Six genetically identified strains of *M. gordonae* were biochemically misidentified as *M. scrofulaceum* because they did not hydrolyze Tween 80. Although *Bergey's Manual of Systematic Bacteriology* describes several biochemical tests to differentiate them, the two species are biochemically closely related (23).

Four strains tentatively identified as *M. fortuitum* were found to belong genetically to *M. chelonae*. One major reason for their misidentification was that they reduced nitrate. As shown in Table 3, nitrate reduction is used to differentiate *M. fortuitum* from *M. chelonae*. Wayne and Kubica (23) also stated that *M. fortuitum* can reduce nitrate but that *M. chelonae* does not reduce nitrate. However, four strains genetically identified as *M. chelonae* subsp. *abscessus* reduced nitrate. Thus, nitrate-positive atypical *M. chelonae* subsp. *abscessus* cannot be differentiated by this set of

characteristics. Strain KPM 750 was biochemically misidentified as *M. chelonae* subsp. *abscessus* because it grew on 0.2% picrate medium. As shown in Table 3, growth on 0.2% picrate medium is the only key characteristic which separates *M. chelonae* subsp. *abscessus* from *M. chelonae* subsp. *chelonae*.

Six strains tentatively identified as *M. nonchromogenicum* complex did not hybridize to any of the three type strains in this complex and also did not hybridize to the DNAs of *M. shimoidei* and *M. malmoense*. In early experiments, we only immobilized the DNAs of the three type strains of the *M. nonchromogenicum* complex; however, *M. shimoidei* and *M. malmoense* should be added to this group of bacteria in future work because they are isolated from human clinical specimens.

Ten biochemically identified but genetically unidentified strains and 15 strains unidentified by both biochemical and genetic methods are being studied further to determine whether they belong to established species or to new species.

In our method, the DNA of a clinical strain is labeled but is not immobilized in microdilution plate wells; thus, the homology between the clinical strain and a reference strain cannot be calculated. We expect that a strain which is genetically independent and not represented among the type strains but highly related to one of the type strains by 60 to 69% homology will be misidentified by our method. Thus, for taxonomic studies, definite homologies should be determined by immobilizing DNAs of both a clinical strain and a type strain. The optimal temperature for DNA-DNA hybridization of *Mycobacterium* species is around 40°C in our solution, as calculated from the guanine plus cytosine contents of the DNAs. However, we used 60°C as the hybridization temperature to increase the stringency of the experiment and to minimize misidentification.

Rapid identification of several *Mycobacterium* species was made possible by the development of species-specific DNA probes (1, 15, 16). These DNA probes are used for identification and detection. It is important to detect *M. tuberculosis* from the specimen directly, without spending the time required for isolation. However, specific DNA probes have only been developed for a few *Mycobacterium* species. To prepare species-specific DNA probes for all of the species described is not practical. The microdilution plate method described here does not require a specific probe. Dried plates containing immobilized DNA can be stored for more than 1 year. Thus, it is possible to identify isolated strains within 3 to 4 h if such DNA plates are readily available. Combinations of specific DNA probe methods for detection and the microdilution plate hybridization method for identification will contribute to the rapid diagnosis and treatment of patients suspected of having mycobacterial infections.

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