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High-Performance Liquid Chromatography Analysis of Mycolic Acids as an Aid in Laboratory Identification of *Rhodococcus* and *Nocardia* Species

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High-performance liquid chromatography analysis of the *p*-bromophenacyl esters of mycolic acids from whole organisms gave chromatographic patterns that were useful in differentiation of *Rhodococcus* and *Nocardia* species. *Rhodococcus equi*, *R. erythropolis*, and *R. rhodochrous* contained more-polar mycolic acids and were easily separated from the less-polar mycolic acid-containing species of *R. sputi*, *R. bronchialis*, *R. corallinus*, *R. rubropertinctus*, and *R. terrae*. The less-polar mycolic acid-containing *Rhodococcus* species showed chromatographic patterns that partially overlapped (in elution times) the patterns of *Nocardia asteroides*, *N. otitidiscaviarum*, and *N. brasiliensis*, but the larger number of peaks in the last species made separation between the genera possible. Distinct chromatographic patterns were found for most species, except for *R. equi* strains that showed two different patterns. Strains of *R. rubropertinctus* and *R. terrae* appeared identical. *N. asteroides* and *N. otitidiscaviarum* showed similar mycolic acid patterns.

Nocardia species are involved in human infection more often than are *Rhodococcus* species, but infrequent infections with *Rhodococcus* species have been reported in immunocompromised hosts (13, 17). Actinomycete infections often are clinically indistinguishable from other infections of bacterial origin. Recognition and diagnosis rely upon isolation and identification of the etiologic agent (9, 20). Accurate identification may be important clinically because *Rhodococcus* and *Nocardia* species are susceptible to different antimicrobial agents (10). *Rhodococcus*, the current taxon name, has been recognized in the past by numerous generic names, including *Nocardia*, *Corynebacterium*, "*Gordona*," and *Mycobacterium* (8, 12). Early confusion in the taxonomic assignment of *Rhodococcus* species has left a degree of uncertainty in methods for their laboratory identification (4, 8, 10). Physiologic characteristics of *Rhodococcus* species have often been confused, and species assignment has been difficult.

Mycolic acids are high-molecular-weight β-hydroxy fatty acids found as stable cell wall components of acid-fast bacteria, i.e., *Corynebacterium*, *Rhodococcus*, *Nocardia*, and *Mycobacterium* spp., and have been useful markers for differentiating these genera (16, 18, 19, 22). It has been shown that mycolic acids from *Corynebacterium*, *Nocardia*, and *Mycobacterium* spp. have carbon chain lengths centered around C_{32}, C_{50}, and C_{80}, respectively (1). Earlier studies of rhodococci demonstrated a heterogeneous group of bacteria with considerable variation in mycolic acid chain lengths. *Rhodococcus* strains of the rhodochrous complex contain mycolic acids with carbon chain lengths of C_{34} to C_{50} (24). Moreover, those *Rhodococcus*-like organisms for which the genus "*Gordona*" was created have mycolic acids with longer chain lengths, i.e., C_{52} to C_{66}, and appear closely related to *Nocardia* species (2).

We previously examined the mycolic acids in *Corynebacterium*, *Nocardia*, *Rhodococcus*, and *Mycobacterium* species by using high-performance liquid chromatography (HPLC) and found the representative mycolic acid patterns useful in assigning a particular organism to one of the four mycolic acid-containing genera (5). In this study, we show that HPLC analysis of mycolic acids provides supplementary information useful for the species identification of organisms belonging to either the *Rhodococcus* or *Nocardia* genus. Furthermore, *Actinomadura* and *Streptomyces* species, both of which lack mycolic acids, were included as part of the study to show the ease of separating these organisms from *Nocardia* and *Rhodococcus* species.

MATERIALS AND METHODS

Microbial strains and cultivation conditions. Organisms representing nine *Rhodococcus* species, three *Nocardia* species, two *Actinomadura* species, and one *Streptomyces* species were obtained from the American Type Culture Collection, Rockville, Md., and from laboratories at the Centers for Disease Control, Atlanta, Ga. Specifically, the following strains were used: *Rhodococcus rhodochrous* ATCC 4276, Goodfellow 372 (ATCC 13808), and Goodfellow 1022; *R. equi* Goodfellow NCTC 4219, Goodfellow NCTC 1621, Goodfellow 1256, ATCC 6939, and CDC F5849; *R. erythropolis* ATCC 4277 and ATCC 25544; *R. rubropertinctus* ATCC 14352; *R. bronchialis* ATCC 25592; *R. terrae* ATCC 25594; *R. corallinus* ATCC 25593; *R. sputi* ATCC 29627; *Nocardia asteroides* ATCC 19247, CDC 84-041085 (89), CDC 84-041618 (105), and CDC B1042; *N. brasiliensis* ATCC 19296; *N. otitidiscaviarum* (*N. caviae*) ATCC 14629 and CDC 738; *Actinomadura madurae* ATCC 19425; *A. pelletieri* ATCC 14816; and *Streptomyces somaliensis* ATCC 33201. The Goodfellow cultures were obtained from Ruth Gordon (Institute of Microbiology, Rutgers University, State University of New Jersey, New Brunswick). Bacteria were grown on agar slants prepared with tryptone (1.0%), yeast extract (0.5%), and glycerin (1.0%). Incubation was at 28°C for 5 to 7 days.

Saponification conditions. Bacteria were harvested with a wire loop (1 to 2 loopsful) and placed directly into 2 ml of saponification reagent (5% potassium hydroxide in 50%

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TABLE 1. Characteristics of mycolic acid peaks for 11 species

Peak no.[a]	Δt (SEM)[b] for peaks observed in:											
	R. equi		*R. erythropolis*	*R. rhodochrous*	*R. terrae*[c]	*R. rubropertinctus*[c]	*R. corallinus*[c]	*R. bronchialis*[c]	*R. sputi*[c]	*N. asteroides*	*N. otitidiscaviarum*	*N. brasiliensis*[c]
	Early	Late										
1	−4.37 (0.08)[c]	−9.13 (0.11)	−5.93 (0.08)	−4.29 (0.04)	−11.39	−11.39	−15.14	−13.06	−0.79	−2.58 (0.22)	−3.11 (0.08)	−4.45
2	−3.92 (0.07)	−8.07 (0.12)	−5.41 (0.07)	−3.62 (0.008)	−11.00	−11.02	−14.21	−12.61	−0.36	−2.08 (0.21)	−2.60 (0.05)	−3.86
3	−3.38 (0.07)	−7.30 (0.07)	−4.45 (0.05)	−3.30 (0.25)	−9.72	−9.71	−13.69	−12.21	0	−1.64 (0.13)	−2.00 (0.05)	−3.41
4	−2.90 (0.05)	−6.40 (0.01)	−4.11 (0.02)	−2.75 (0.03)	−8.15	−8.17	−12.52	−10.41	+0.40	−1.36 (0.12)	−1.54 (0.04)	−2.89
5	−2.42 (0.05)	−6.03 (0.01)	−3.52 (0.03)	−2.11 (0.02)	−6.66	−6.71	−12.27	−9.64	+0.76	−0.85 (0.07)	−0.97 (0.03)	−2.43
6	−1.92 (0.04)	−5.33 (0.07)	−2.57 (0.04)	−1.75 (0.06)	−1.65	−1.83	−11.69	−8.25	+1.16	−0.46 (0.06)	−0.51 (0.02)	−1.98
7	−1.47 (0.03)	−4.28 (0.05)	−2.31 (0.01)	−1.32 (0.01)	−1.20	−1.22	−10.84	−7.19	+1.50	0	0	−1.52
8	−0.93 (0.01)	−3.67 (0.03)	−1.70 (0.01)	−0.69 (0.008)	−0.89	−0.89	−10.46	−1.05	+1.90	+0.41 (0.02)	+0.45 (0.01)	−1.14
9	−0.53 (0.01)	−3.22 (0.10)	−0.63 (0.03)	0	−0.33	−0.36	−9.86	−0.75		+0.89 (0.03)	+0.91 (0.03)	−0.70
10	0	−2.69 (0.04)	0	+0.62 (0.003)	0	0	−9.22	−0.32		+1.31 (0.05)	+1.36 (0.04)	−0.36
11	+0.39 (0.01)	−2.07 (0.07)	+0.93 (0.03)	+1.22 (0.008)	+0.46	+0.46	−8.42	0		+1.76 (0.04)	+1.77 (0.05)	0
12	+0.92 (0.02)	−1.65 (0.09)	+1.58 (0.005)	+1.82 (0.01)	+0.83	+0.82	−7.88	+0.36		+2.16 (0.05)	+2.21 (0.06)	+0.69
13	+1.80 (0.02)	−1.28 (0.02)	+2.36 (0.02)	+2.29 (0.07)	+1.25	+1.25	−7.68	+0.70				+1.08
14	+2.15 (0.03)	−0.57 (0.01)	+3.02 (0.005)	+2.93 (0.02)	+1.60	+1.59	−7.23	+1.07				+1.54
15	+2.64 (0.03)	0	+3.68 (0.01)				−6.96					
16	+2.97 (0.03)	+0.59 (0.02)	+4.32 (0.005)				−6.57					
17	+3.46 (0.04)	+1.19 (0.01)	+4.91 (0.01)				−6.23					
18	+3.78 (0.04)	+1.76 (0.03)	+5.52 (0.005)				−5.57					
19	+4.54 (0.04)	+2.32 (0.01)	+6.05 (0.02)				−1.44					
20		+2.88 (0.03)					−0.83					
21							−0.50					
22							0					
23							+0.38					
24							+0.82					
25							+1.19					
26							+1.57					

[a] Peak number indicates order of elution and not identity between species.

[b] Δt (SEM), mean time difference (standard error of the mean) between emergence of major (tallest) mycolic acid peak, designated 0 (which acted as an internal standard for the species), and all other mycolic acid peaks of the species. A positive value indicates that the peak appeared after the major peak, while a negative value means that the peak preceded the major peak.

[c] Data for single isolates included here only for comparison with other species.

TABLE 2. HPLC criteria for species identification

Species	Mycolic acid peaks				
	No. of groups	No.		Range (min)	
		Group 1 or only group	Group 2	Group 1 or only group	Group 2
R. equi (early)	1	20		10.72–19.62	
R. equi (late)	1	20		12.64–24.66	
R. erythropolis	1	19		12.35–24.32	
R. rhodochrous	1	14		17.72–24.94	
R. terrae	2	5	9	14.54–19.27	24.28–27.53
R. rubropertinctus	2	5	9	14.70–19.38	24.26–27.68
R. corallinus	2	18	8	10.70–20.27	24.40–27.41
R. bronchialis	2	7	7	14.06–19.93	26.07–28.19
R. sputi	1	8		26.07–28.76	
N. asteroides	1	12		22.67–27.41	
N. otitidiscaviarum	1	12		21.91–27.23	
N. brasiliensis	1	14		23.88–29.87	

ethanol). Saponification was completed overnight at 85°C in Teflon-capped 13-mm tubes. After saponification, the mixture was cooled to room temperature and then acidified with 0.5 ml of 6 N hydrochloric acid, followed by the addition of 2 ml of chloroform. The suspension was thoroughly mixed and then centrifuged to separate the chloroform layer (15). The chloroform fraction that contained the fatty acids was added to a 5-ml Reacti-Vial (Pierce Chemical Co., Rockford, Ill.) and evaporated to dryness, and the residue was suspended in 0.5 ml of chloroform and derivatized.

Derivatization conditions. Chloroform-extracted mycolic acids were derivatized to their *p*-bromophenacyl esters by reaction with an α,*p*-bromophenacyl bromide and dicyclohexyl-18-crown-6-ether (7). The reagents, including instructions for use, were purchased in a kit from Alltech Associates, Inc., Applied Science Div., State College, Pa. Derivatization was at 85°C for 40 min with a Reacti-Therm heating and stirring module (Pierce Chemical Co.). After derivatization, the chloroform was evaporated, and the derivatized product was suspended in 100 μl of chloroform.

HPLC. Analysis of mycolic acids was carried out by HPLC with a Beckman model 450 data system controller (Beckman Instruments, Inc., Berkeley, Calif.) equipped with an Altex model 155 UV detector adjusted to a fixed wavelength of 254 nm. A 5-μm particle size, C18 reverse-phase column (4.6 mm by 22 cm) (Pierce Chemical Co.) was equilibrated in 90% acetonitrile–10% chloroform. After injection of a 5-μl sample, gradient elution conditions were established by two Beckman model 110A solvent pumps. The solvent concentration was changed linearly to 40% acetonitrile–60% chloroform over a period of 30 min at a flow rate of 1.5 ml/min.

Sample screening. Initially, samples were screened to establish if they belonged to either the *Rhodococcus* or *Nocardia* genus by a modification of a previously reported procedure (5) as follows. The column was equilibrated in 5% chloroform–95% methanol for 5 min, and then the solvent concentration was changed to 25% chloroform over 5 min, followed by a linear gradient to 70% chloroform over 30 min at a flow rate of 1.5 ml/min.

RESULTS

Reproducibility of chromatographic patterns was demonstrated for different strains of the same species. Results for *Rhodococcus* and *Nocardia* strains that were studied are shown in Table 1.

Genus assignment with the modified screening program (chloroform-methanol) was based on peak retention time and, although not shown here, this was described in detail earlier (5); e.g., peaks emerging before 5.3 min were nonmycolic acid fatty acids, solvent, and derivatization reagents, whereas peaks from 5.3 to 8.5 min were corynomycolic acids. For the present study, peaks emerging beyond 10 min were used to identify species within the genera *Rhodococcus* and *Nocardia* (Table 2). Peaks emerging from 10.72 to 24.94 min were short-chain mycolic acids representative of *Rhodococcus* species. Peaks emerging from 21.91 to 29.87 min were nocardomycolates. An intermediate group in the genus *Rhodococcus* had long-chain mycolic acids with retention times from 14.54 to 28.76 min. Differences between species within this intermediate group will be detailed later. Strains of *Actinomadura* and *Streptomyces* contained no mycolic acids and had no peaks in any mycolic acid region (data not shown).

Examination of five strains of *R. equi* by the gradient system of chloroform and acetonitrile revealed two different chromatographic patterns. Polar mycolic acids of one pattern eluted within a range of 10.72 to 19.62 min with a major peak at 15.09 min (Fig. 1A). The other less polar pattern had a longer elution range of 12.64 to 24.66 min with a major peak at 21.84 min (Fig. 1B). *R. rhodochrous* had an elution range of 17.72 to 24.94 min and a retention time for its major peak of 22.03 min (Fig. 1D). The longer elution range of *R. equi* (Fig. 1B) overlapped the peaks of *R. erythropolis* that had an elution range of 12.35 to 24.32 min (Fig. 1C). There was a definite difference in the retention time of the major peak for *R. erythropolis*, 18.82 min, as well as obvious differences in Δt values for other peaks surrounding the major peak (Table 1).

R. terrae and *R. rubropertinctus* had almost identical chromatographic patterns with an elution range of 14.54 to 27.53 min and a major peak at 25.93 min for the former and a range of 14.70 to 27.53 min and a major peak of 26.09 min for the latter, respectively (Fig. 1E and F). A close relationship between *R. terrae* and *R. rubropertinctus* was also reflected in the total number of peaks (14 for both) and in almost identical Δt values (Table 1). The group of early peaks (14.54 to 19.38 min) for both these species was sometimes difficult to distinguish from background noise, but the important peaks are identified by Δt position in Table

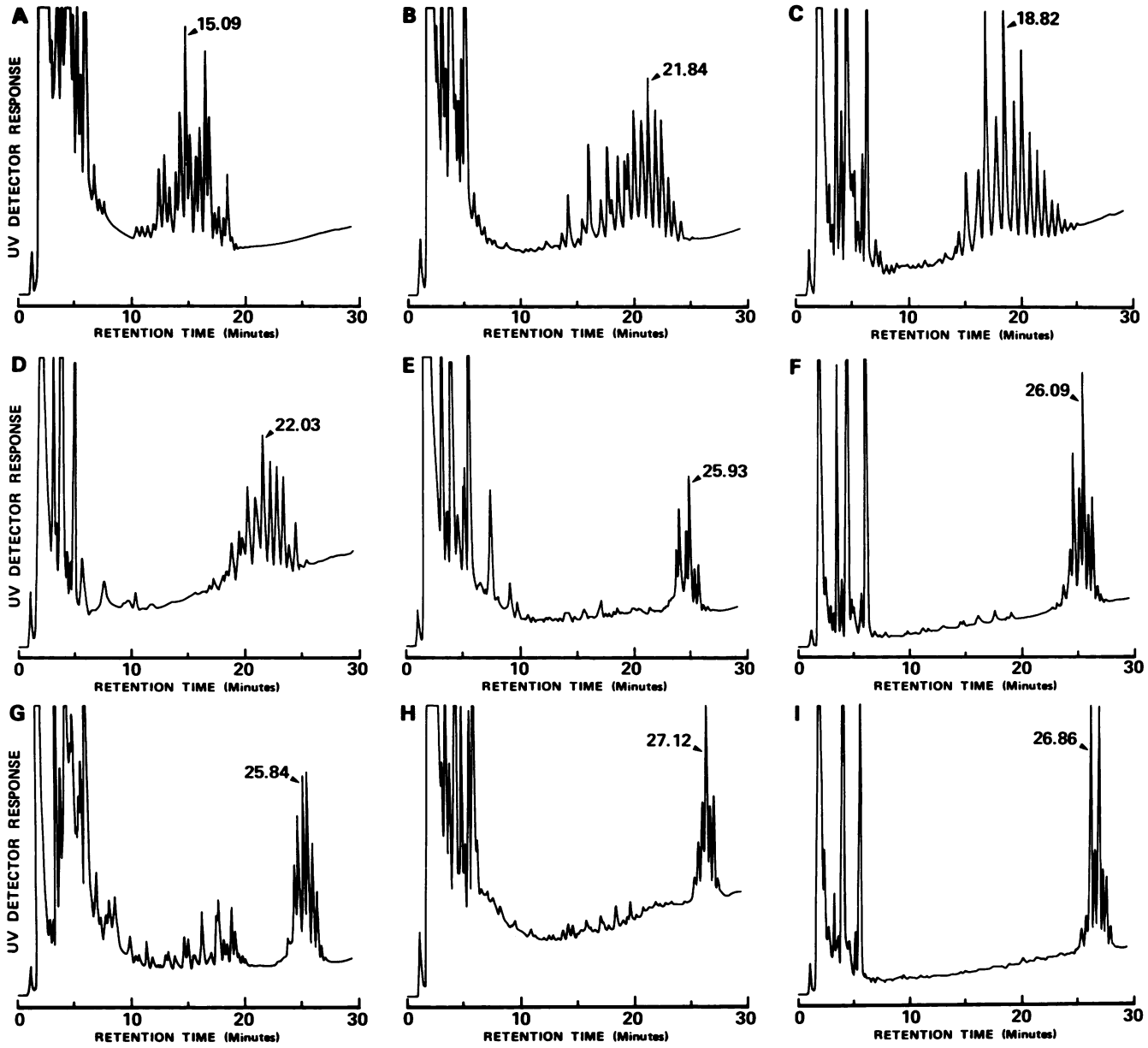

FIG. 1. Fractionation patterns of mycolic acids as *p*-bromophenacyl esters from *Rhodococcus* species. (A) *R. equi* NCTC 1621; (B) *R. equi* NCTC 4219; (C) *R. erythropolis* ATCC 25544; (D) *R. rhodochrous* ATCC 4276; (E) *R. terrae* ATCC 25594; (F) *R. rubropertinctus* ATCC 14352; (G) *R. corallinus* ATCC 25593; (H) *R. bronchialis* ATCC 25592; (I) *R. sputi* ATCC 29627. Peaks emerging before 10 min were excess derivatization reagents, solvent, and short-chain cellular fatty acids.

1. *R. corallinus* had a similar elution range of 10.70 to 27.41 min and a major peak at 25.84 min, but the peak patterns were not identical (Table 1). Additionally, *R. corallinus* also displayed a prominent group of 18 early eluting peaks from 10.70 to 20.27 min (Fig. 1G). *R. bronchialis* and *R. sputi* had the least-polar mycolic acids, with retention times ranging from 26.07 to 28.19 min (major peak, 27.12 min) and 26.07 to 28.76 min (major peak, 26.86 min), respectively. Although similar overall retention times were observed, neither the general appearance of the chromatographic patterns (Fig. 1H and I) nor the Δt values (Table 1) were the same.

N. asteroides, *N. otitidiscaviarum*, and *N. brasiliensis* had fractionation patterns that partially overlapped the *Rhodococcus* patterns. Except for *R. sputi*, the long-chain mycolic acid-containing *Rhodococcus* species were distinguished by a minor group of early eluting peaks and a major second group of peaks. Additionally, these *Rhodococcus* species contained 7 to 9 peaks in their second major peak group, whereas the *Nocardia* species had 12 to 14 peaks in a single, congregate peak group (Table 2). *N. brasiliensis* had the greatest range, 23.88 to 29.87 min and a major peak at 28.33 min (Fig. 2C). *N. asteroides* had retention times of 22.67 to 27.41 min and a major peak of 25.41 min (Fig. 2A). The chromatographic pattern of *N. otitidiscaviarum* had the same number of peaks and similar retention times as *N. asteroides* (Fig. 2B).

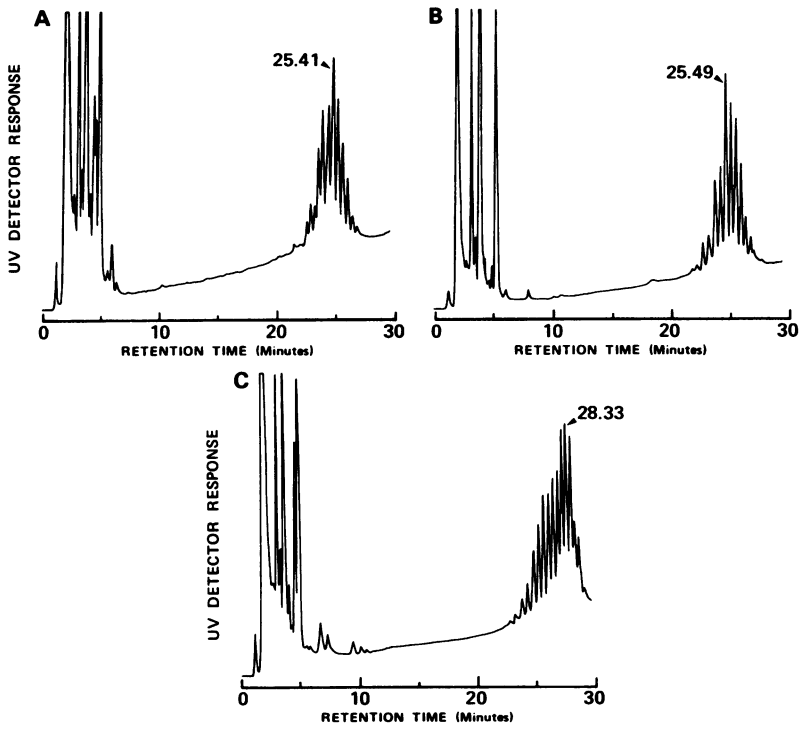

FIG. 2. Fractionation patterns of mycolic acids as *p*-bromophenacyl esters from *Nocardia* species. (A) *N. asteroides* ATCC 19247; (B) *N. otitidiscaviarum* ATCC 14629; (C) *N. brasiliensis* ATCC 19296.

DISCUSSION

The HPLC chromatographic patterns of *p*-bromophenacyl esters of mycolic acids from *Rhodococcus* and *Nocardia* species are helpful in species characterization. It has been shown by definitive thin-layer chromatography-mass spectrometry and gas chromatography-mass spectrometry that the mycolic acid content of certain mycolic acid-containing bacteria is species specific (19, 24). Although, no attempt was made to analyze individual peaks produced by HPLC, it is likely that each peak represents mixtures of mycolic acids (25).

Preliminary testing of mycolic acid esters by the HPLC screening program with chloroform and methanol usually enabled us to separate one genus from another. There was a partial overlap of the 12 to 14 mycolic acid peaks from the *Nocardia* species with the second group of 7 to 9 mycolic acid peaks from the long-chain mycolic acid-containing *Rhodococcus* species, but the difference in the total numbers of peaks enabled the separation of the genera. This partial overlap was expected, as it has been reported that *Nocardia* species have mycolic acids of intermediate carbon chain length (C_{46} to C_{60}) compared with the short-chain mycolic acid-containing *Rhodococcus* (C_{34} to C_{50}) and the long-chain mycolic acid-containing *Rhodococcus* (C_{52} to C_{66}) species (1).

Rhodococcus species could easily be grouped into short- and long-chain mycolic acid-containing groups by HPLC. Representative of the short-chain class were *R. equi*, *R. erythropolis*, and *R. rhodochrous*. Species of *R. rubropertinctus*, *R. corallinus*, *R. terrae*, *R. bronchialis*, and *R. sputi* represented the long-chain mycolic acid group.

Extracts of *R. equi* strains yielded two different chromatographic patterns. *R. equi* was the only species examined for which a strain variation was found in the HPLC patterns. *R. equi* has been shown to contain short-chain mycolic acids (C_{30} to C_{38}), is similar to *Corynebacterium* spp., and is sometimes erroneously referred to in the literature as *Rhodococcus* (*Corynebacterium*) *equi* or *C. hoagii* (3, 4, 6, 11). The strain variation in HPLC pattern that we observed may be a reflection of this taxonomic confusion.

The HPLC patterns could be used to distinguish the less-polar mycolic acid-containing species of *R. sputi*, *R. bronchialis*, and *R. corallinus*, and to separate them from *R. rubropertinctus-R. terrae*. However, it was not possible to distinguish *R. rubropertinctus* from *R. terrae*. Previous reports have also noted similarities between these two species, and it has been suggested that *R. terrae* is a variety of the species *R. rubropertinctus* (23). Also, examination of the mycobactins from *R. terrae* and *R. rubropertinctus* by thin-layer chromatography and HPLC have shown almost identical patterns (14). However, the DNA-DNA homology data conflict with these results and indicate that *R. terrae* and *R. rubropertinctus* are separate species and that *R. rubropertinctus* and *R. corallinus* are synonyms (21).

The mycolic acid peaks from *Nocardia* species had patterns with retention times similar to those of some species of *Rhodococcus*. Although *N. asteroides* showed a pattern similar to that of *N. otitidiscaviarum*, further quantitative analysis may show subtle differences. *N. brasiliensis* has been characterized by mass spectrometry as containing mycolic acids with carbon chain lengths of C_{52} to C_{60} (1). This longer chain length was reflected in longer retention times for *N. brasiliensis*.

HPLC is a simple and rapid procedure for the analysis of mycolic acids found in *Rhodococcus* and *Nocardia* species, and this study suggests its usefulness for chemotaxonomic purposes. Furthermore, the lack of mycolic acids in *Actinomadura* and *Streptomyces* species enables one to separate them from the closely related genus *Nocardia*. Because of

differences in instrument hardware, investigators must standardize their systems to obtain reproducible and differential patterns.

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