Composition of Mycolic Acid Molecular Species as a Criterion in *Nocardial* Classification

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By using gas chromatography and gas chromatography-mass spectrometry, we analyzed the mycolic acid compositions of 18 strains of *Nocardia asteroides*, 17 strains of *Nocardia farcinica*, and 17 strains of *Nocardia nova* classified by numerical taxonomy. These organisms had characteristic mycolic acid compositions. We calculated the peak areas of the molecular species of mycolic acids on gas chromatograms and determined the average total carbon number in each strain. The strains of *N. asteroides* were divided into five groups, and the type strain belonged to group C₅₄. The strains of *N. farcinica* were divided into three groups, and the type strain was in group C₅₃. On the other hand, the strains of *N. nova* differed distinctly from the other two species and belonged mainly to groups C₅₆ and C₅₇. Our detailed analysis of mycolic acids was simple and precise. Therefore, the use of this method should be encouraged more for nocardial classification in combination with DNA or RNA analysis.

For a long time classification of the genus *Nocardia* has been a major issue in the taxonomy of the order *Actinomycetales*. In particular, the chemotaxonomy of *Nocardia asteroides* and related species has been confused for decades. Goodfellow stated that *Nocardia farcinica* was a good taxospecies, but *N. asteroides* was markedly heterogeneous based on numerical taxonomy data (9, 26). After this, a new species, *Nocardia nova*, was differentiated from a subgroup of *N. asteroides*, was proposed by Tsukamura et al. (27), and was characterized on the basis of numerical (27) and chemotaxonomic (31) analysis data. The taxonomy of *N. asteroides* and *N. farcinica* was still a difficult issue. Recently, the yeast killer system (20), ribotyping encoded by 16S rRNA (6, 16), antibiotic sensitivity (28), and opacification of Middlebrook agar (8) have been used to differentiate these three *Nocardia* species.

Mycolic acids are unique high-molecular-weight 3-hydroxyl fatty acids with long alkyl chains at position 2 and are characteristic cell wall constituents of *Mycobacterium* (3, 4, 11, 12, 14, 17), *Nocardia* (2, 30), *Rhodococcus* (1, 2), *Gordonia* (1), *Corynebacterium* (7, 29), *Tsukamurella* (10), and *Dietzia* (21) species. Since the mycolic acid subclass or molecular species compositions vary greatly among taxa, mycolic acids are good markers for differentiating genera or species. However, characteristics used for classification in most previous studies were total carbon number, the number of double bonds, and the acids released by pyrolysis of mycolic acids. Previously, we developed an analytical method for whole mycolic acids without pyrolysis in which trimethylsilylated (TMS) ether derivatives were used for gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) (3, 4, 11, 12, 29, 30). We utilized the average carbon number and the length of the α -branch obtained by this method to classify taxa.

We have many *Nocardia* strains which were classified as *N. asteroides*, *N. farcinica*, and *N. nova* strains by numerical methods by M. Tsukamura. Since there is strong evidence that *N. asteroides* is heterogeneous and that *N. asteroides* is closely related to *N. farcinica*, we investigated the mycolic acid com-

positions of more than 50 strains belonging to *N. asteroides*, *N. farcinica*, and *N. nova* in order to classify them on the basis of chemical taxonomy data.

MATERIALS AND METHODS

All of the *Nocardia* strains were divided preliminarily on the basis of the results of a numerical analysis (27) and were supplied by M. Tsukamura, National Chubu Hospital (Obu, Aichi, Japan). The *Nocardia* strains which we used are shown in Table 1. Each strain was grown in medium containing 1% glucose, 0.5% peptone, and 0.2% yeast extract with the pH adjusted to 7.2 at 37°C for 3 to 7 days on a rotary shaker.

Mycolic acids were extracted and separated as described previously (4). α -Mycolic acid methyl esters were separated by preparative thin-layer chromatography on silica gels and were trimethylsilylated with *N,O*-bis-(trimethylsilyl)trifluoroacetamide-pyridine (1:2, vol/vol) at 80°C for 30 min.

The numbers of carbon atoms and double bonds were determined by GC and GC-MS. To separate each molecular species of mycolic acid that differed in carbon number, GC was performed with a Shimadzu model GC-9A instrument equipped with a column packed with 1% OV-1 or a capillary column (0.53 mm by 15 m) coated with 5% methyl phenyl silicone. The column temperature was programmed to increase from 300 to 330°C at a rate of 1°C/min. Mass spectrometry and mass chromatography were carried out with a Hitachi model M-80 instrument. The ion source energy was 20 eV, and the accelerating voltage was 3 kV. Detailed conditions have been described previously (11). Every analysis was performed at least in duplicate. The peak areas of molecular species were calculated by the vertical division method. The average total carbon numbers of mycolic acids were determined from the sum of the products of the carbon numbers multiplied by the percent compositions.

RESULTS

GC profiles of TMS methyl mycolates of strains of *Nocardia* species. The total fatty acid methyl esters from each *Nocardia* species produced two spots on thin-layer chromatograms. The upper spot corresponded to nonpolar fatty acid methyl esters. The lower spot, with an *R_f* value of 0.5 to 0.6, corresponded to α -mycolic acid esters. Thus, the *Nocardia* species, with one subclass of mycolic acids (α -mycolic acids), differed from *Mycobacterium* species.

α -Mycolic acid methyl esters were converted to TMS ether derivatives and were analyzed by GC or GC-MS. Figure 1 shows typical gas chromatograms of TMS ether derivatives of α -mycolic acid methyl esters from the *N. asteroides*, *N. farcinica*, and *N. nova* strains. Each strain produced six to nine peaks corresponding to C₄₀ to C₆₂ mycolic acids, as judged from the mass spectrometric analysis and the relationship between log retention time and carbon number on gas chromato-

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TABLE 1. *Nocardia* strains used in this study

Laboratory no.	Designation(s)	Source
<i>N. asteroides</i> strains		
23008	M123	M. Tsukamura ← I. Uesaka ← N. M. McClung
23019	M78	M. Tsukamura ← I. Uesaka ← N. M. McClung
23046	M129	M. Tsukamura ← I. Uesaka ← N. M. McClung
23049	M132	M. Tsukamura ← I. Uesaka ← N. M. McClung
23064	M161	M. Tsukamura ← I. Uesaka ← N. M. McClung
23094	ATCC 23824, R399	M. Tsukamura ← R. E. Gordon
23099	R860	M. Tsukamura ← R. E. Gordon
23169	N9	M. Tsukamura ← K. P. Schaal
23170	N10	M. Tsukamura ← K. P. Schaal
23172	N14	M. Tsukamura ← K. P. Schaal
23173	N15	M. Tsukamura ← K. P. Schaal
23175	N57	M. Tsukamura ← K. P. Schaal
23182	N13	M. Tsukamura ← M. Goodfellow
23183	N317	M. Tsukamura ← M. Goodfellow
23189	N669	M. Tsukamura ← M. Goodfellow
23203		M. Tsukamura; sputum, human
23205		M. Tsukamura; sputum, human
23206 ^T	ATCC 19247 ^T	M. Tsukamura ← I. J. Bousfield
<i>N. farcinica</i> strains		
23004	M72	M. Tsukamura ← I. Uesaka ← N. M. McClung
23013	M146	M. Tsukamura ← I. Uesaka ← N. M. McClung
23055	M146	M. Tsukamura ← I. Uesaka ← N. M. McClung
23087	ATCC 23826, M205	M. Tsukamura ← I. Uesaka ← N. M. McClung
23101	W3409B	M. Tsukamura ← R. E. Gordon
23102	ATCC 3318, R3318	M. Tsukamura ← R. E. Gordon
23157 ^T	ATCC 3318 ^T	M. Tsukamura ← M. P. LeChevalier
23162		M. Tsukamura; pleural pus, human
23163		M. Tsukamura; pleural pus, human
23164		M. Tsukamura; sputum, human
23165		M. Tsukamura; sputum, human
23166	N1	M. Tsukamura ← K. P. Schaal
23167	N6	M. Tsukamura ← K. P. Schaal
23168	N7	M. Tsukamura ← K. P. Schaal
23171	N12	M. Tsukamura ← K. P. Schaal
23201		M. Tsukamura; bronchial secretion, human
E12618		M. Tsukamura ← H. Shimode; human
<i>N. nova</i> strains		
23006	M93	M. Tsukamura ← I. Uesaka ← N. M. McClung
23012	M145	M. Tsukamura ← I. Uesaka ← N. M. McClung
23032	M10	M. Tsukamura ← I. Uesaka ← N. M. McClung
23038	M93	M. Tsukamura ← I. Uesaka ← N. M. McClung
23044	M124	M. Tsukamura ← I. Uesaka ← N. M. McClung
23088	M206	M. Tsukamura ← I. Uesaka ← N. M. McClung
23095 ^T	ATCC 33726 ^T , R443 (1) ^T	M. Tsukamura ← R. E. Gordon
23096	R443 (2)	M. Tsukamura ← R. E. Gordon
23103	ATCC 9970	M. Tsukamura ← R. E. Gordon
23174	N23	M. Tsukamura ← K. P. Schaal
23177	N229	M. Tsukamura ← K. P. Schaal
23181	E9	M. Tsukamura ← M. Goodfellow
23192	N692	M. Tsukamura ← M. Goodfellow
23196	N878	M. Tsukamura ← M. Goodfellow
23204	ATCC 33727	M. Tsukamura; sputum, human
23209	ATCC 33726	M. Tsukamura; human
23210	ATCC 33727	M. Tsukamura; human

grams. As shown in Fig. 1, the largest peak for strain 23206, the type strain of *N. asteroides*, was the C₅₄ peak. On the other hand, the gas chromatogram obtained for strain 23094 revealed that C₅₂ was the main component. The chromatogram obtained for strain 23064 contained two major mycolic acid peaks (C₅₂ and C₅₄). The mycolic acids of strains 23099 and 23019 differed completely from the mycolic acids of other *N. asteroides* strains, and the most abundant components were

around C₄₈ and C₄₄, respectively. On the other hand, for the type strain of *N. farcinica*, 23157, the most abundant species of mycolic acids was C₅₄ on gas chromatograms, and the secondary peak was C₅₂. Many other *N. farcinica* strains produced almost the same GC profile as strain 23157^T. For another *N. farcinica* strain tested, strain 23013, the major peak was C₅₂, and the secondary peak was C₅₄. In contrast to *N. asteroides* and *N. farcinica*, the mycolic acid profiles of all of the *N. nova*

FIG. 1. GC analysis of TMS methyl mycolates from several strains of three *Nocardia* species (*N. asteroides*, *N. farcinica*, and *N. nova*). The number for each peak indicates the total number of carbon atoms in the mycolic acid.

tested (including 23095^T and 23088) revealed that C₅₆ and C₅₈ mycolic acids were the most abundant components.

Differentiation of three *Nocardia* species based on the total carbon and double bond numbers of mycolic acids. GC-MS revealed the more detailed structures of the individual mycolic acid molecules. Figure 2 shows mass spectra of TMS derivatives of methyl mycolates of *N. asteroides* 23094, *N. farcinica* 23190, and *N. nova* 23177. As Fig. 3 shows, general fragmentation of TMS methyl mycolates during electron impact mass spectrometry yielded characteristic ions useful for identification. The molecular ion [M]⁺ was usually weak, but [M-15]⁺ ions (loss of CH₃) were generally abundant and could be used to establish molecular weight. Fission of the intraalkyl chain (C₂-C₃) gave the fragment ion [A]⁺. This ion was useful for determining the straight-chain alkyl moieties of the original mycolic acids. In contrast, cleavage between C₃ and C₄ resulted in fragment ion [B]⁺, which was useful for identifying the α -branched chain structure of mycolic acids. In *N. asteroides*, the presence of [M-15]⁺ corresponding to C₄₄ to C₅₈ mycolic acids having zero to three double bonds was clearly demonstrated, and the mycolic acids with longer chains tended to have more double bonds. For C₅₂ mycolic acids, the most abundant [M-15]⁺ ions were observed at *m/e* 843 and 845, corresponding to C_{52:2} and C_{52:1} mycolates, respectively. A similar [M-15]⁺ ion was observed in the C₅₂ mycolate of *N. farcinica*, and in the C₅₄ mycolate of the same organism, the [M-15]⁺ ion was shifted up by 28 mass units to *m/e* 871 due to C_{54:2}. On the other hand, in *N. nova*, the major mycolic acid molecules had another double bond in the α -branch. The most abundant molecular component was C₅₆ or C₅₈ mycolate and was a mixture of triene and tetraene subclasses.

Comparison of the detailed molecular structures of the mycolic acids of three *Nocardia* species. Another characteristic of the mycolic acids was the presence of both a straight-chain alkyl unit and an α -branched chain in the three *Nocardia* species. As shown in Fig. 2, a certain mycolic acid of *N. asteroides* usually consisted of a mixture of two or more molecules differing in the α -alkyl branch and the straight-chain unit. In the C₅₂ mycolate of *N. asteroides*, at least two kinds of [A]⁺ fragment ions were clearly found at *m/e* 591 and 617 due to C_{36:1} and C_{38:2} straight alkyl chains, respectively, and also two [B]⁺ fragment ions were at *m/e* 371 and 343 due to C_{16:0} and C_{14:0} α -alkyl branches, respectively. Therefore, the C₅₂ mycolate of *N. asteroides* was a mixture of a C_{36:1} straight chain with a C_{16:0} α -branch and a C_{38:2} straight chain with a C_{14:0} α -branch. Similar results were obtained for the C₄₈ to C₅₄ mycolates of *N. asteroides*. In contrast, in the case of *N. farcinica*, the [A]⁺ fragment ion of each mycolate produced a single peak, and therefore the [B]⁺ fragment ion was also a single compound. Thus, the C₅₂ mycolate of *N. farcinica* was a simple molecule with a C_{36:1} (C_{36:2}) straight chain and a C_{16:0} α -branch. On the other hand, we also noted that the molecular species composition of *N. nova* was much more complicated than the molecular species compositions of *N. asteroides* and *N. farcinica*. As shown in Fig. 2, C₅₈ mycolate (C_{58:3} and C_{58:4}) was a mixture of molecular species in at least four combinations (C_{40:2} plus C_{18:1}, C_{40:3} plus C_{18:0}, C_{42:3} plus C_{16:0}, and C_{40:3} plus C_{18:1}). However, we especially noted that the *N. nova* mycolates had abundant C_{18:1} (and C_{18:0}) α -alkyl branches. Thus, they differed from the mycolates of *N. asteroides* and *N. farcinica*.

Grouping based on molecular species composition of mycolic acids in the genus *Nocardia*. To determine the composi-

FIG. 2. Mass spectra of TMS derivatives of methyl mycolates of *N. asteroides* 23094, *N. farcinica* 23190, and *N. nova* 23177.

tions of mycolic acid molecular species, we calculated the peak areas on gas chromatograms. The ratio of each molecular species to the total mycolic acids is shown in Table 2. The largest peaks accounted for more than 25% (nearly 30%) of the total area. The average total carbon numbers for mycolic acids are also shown in Table 2. All of the strains were classified on the basis of the average total carbon number in the mycolic acids of the strains belonging to the same species (Table 3). Eighteen strains of *N. asteroides* identified by numerical taxonomy were separated into the following five groups: groups $C_{\leq 50}$, C_{52} , C_{53} , C_{54} , and C_{55} . Similarly, 17 strains of *N. farcinica* were separated into three groups, groups C_{52} , C_{53} , and C_{54} . Type strain 23157 of *N. farcinica* was in-

cluded in group C_{53} , and *N. asteroides* 23206^T was in group C_{54} . Seventeen strains of *N. nova* were also separated into three groups, groups C_{55} , C_{56} , and C_{57} . All of the *N. nova* strains contained C_{56} or C_{58} mycolic acid as the most abundant component.

DISCUSSION

We analyzed the mycolic acid compositions of more than 50 strains, including the type strains of *N. asteroides*, *N. farcinica*, and *N. nova*, by GC and GC-MS and tried to classify the strains based on their compositions.

Eighteen strains of *N. asteroides* were divided into five

FIG. 3. General fragmentation of TMS derivatives of methyl mycolate during electron impact mass spectrometry.

groups on the basis of the average total carbon numbers (Table 3). Strains 23019 and 23099, members of group C_{50} , were obviously different from other *N. asteroides* strains, *N. farcinica*, and *N. nova*, as shown by the patterns on gas chromatograms (Fig. 1) and by the major components of their mycolic acids (Table 2). The mycolic acids of *Rhodococcus* strains contained 34 to 50 carbon atoms (2). Our previous data for *Rhodococcus* strains revealed a range of 28 to 46 carbon atoms (2). Therefore, it seemed that strains 23019 and 23099 belonged to the genus *Rhodococcus* even though they were classified as *N. asteroides* strains by the numerical method. This implies that classification by the numerical method alone is not accurate and that the original taxon *N. asteroides* was heterogeneous. Tomiyasu (25) described the mycolic acid compositions of three strains of *N. asteroides* (23006, 23007, and 23094) and showed that these compositions differed distinctly. The type strain of *N. asteroides* (23206) belonged to group C_{54} in this study. The matching coefficient for strain 23206^T with strains 23094 (group C_{52}) and 23046 (group C_{53}) was 90% according to previous data (26, 27, 31). The value for the species was also

rather low. The level of DNA complementarity for strains 23206^T and 23094 was as low as 18% (13). The results of DNA amplification with restriction endonuclease analysis (28) and ribotyping (16) also demonstrated that *N. asteroides* strains should be divided into two groups. Immunodiffusion studies revealed that the type strain of *N. asteroides* (ATCC 19247) was clearly distinct from most of the strains of this species (22). Although *N. asteroides* can be identified by various methods and is thought to be an obviously independent *Nocardia* species, it is still heterogeneous on the basis of mycolic acid composition and other evidence.

We showed previously that matching coefficients for *N. farcinica* strains were greater than 92% (31), and other authors have also found that *N. farcinica* is a fairly homogeneous taxon (9, 19). Eighteen strains of *N. farcinica* were separated into three groups based on mycolic acid composition. Group C_{53} of *N. farcinica*, including type strain 23157, contained C_{54} mycolic acid as a major component. The results (13) of previous DNA relatedness studies showed that the levels of relatedness among the strains belonging to group C_{53} were high. Therefore, group C_{53} is probably representative of *N. farcinica*.

We noticed that the groups identified on the basis of average total carbon numbers in mycolic acids overlapped between *N. asteroides* and *N. farcinica*, although the type strain groups were different. The results of a DNA reassociation study indicated that the *N. asteroides* strains fell into three DNA homology groups, one of which contained the type strain of *N. farcinica* (18). A phylogenetic analysis of 16S RNA revealed that two sublines could be recognized in the *Nocardia* clade; *N. asteroides* and *N. farcinica* were members of one subline, while *N. nova* was in the other (6). The overlap of mycolic acid groups observed with the *N. asteroides* and *N. farcinica* strains might mean that these two species are closely related. Differentiation between *N. asteroides* and *N. farcinica* by GC of mycolic acids alone was sometimes very difficult, because the differences in the mycolic acid compositions of the two species are very minor. In this study, using packed-column GC-MS, we did not find obvious differences in the major molecular species of mycolic acids between *N. asteroides* and *N. farcinica*; that is,

TABLE 2. Mycolic acid compositions of *Nocardia* strains

Species	Strain	% of total mycolic acids												Avg no. of C atoms in mycolic acids
		~42 C atoms	44 C atoms	46 C atoms	48 C atoms	50 C atoms	52 C atoms	54 C atoms	56 C atoms	58 C atoms	60 C atoms	62 C atoms	64 C atoms	
<i>N. asteroides</i>	23019	37.4	35.5	19.1	8.0									43.6
	23064		0.1	1.6	5.9	15.0	30.7	32.4	12.3	2.1	0.2			52.8
	23094		0.3	4.5	10.9	20.1	26.7	24.3	10.4	2.3	0.4			52.0
	23099	0.6	7.3	12.8	17.4	18.9	16.8	13.5	6.8	2.8				48.4
	23170				1.8	9.1	18.7	33.9	25.7	9.5	2.5			54.8
	23206 ^T		0.1	0.6	3.4	9.0	21.3	27.8	23.7	11.4	2.8			54.1
<i>N. farcinica</i>	23013			2.2	6.7	15.0	29.1	27.9	13.8	2.2	0.4			51.8
	23055			0.2	2.1	7.6	21.6	36.1	26.1	5.5				54.0
	23101		0.6	7.2	12.0	20.2	28.7	23.5	6.4	1.5				51.5
	23157 ^T			1.2	6.9	13.8	24.8	31.9	17.1	4.2	0.8			53.0
	23165		2.1	4.4	6.2	10.1	16.9	23.5	21.5	5.5				53.0
	23171		0.7	3.1	5.3	14.6	28.0	30.3	15.3	4.0	0.5			53.7
<i>N. nova</i>	23012					3.1	7.1	11.7	30.5	30.5	15.3	2.0		56.8
	23088				0.8	1.9	6.2	21.7	31.3	26.1	12.0			56.3
	23095 ^T				0.4	2.5	5.6	10.8	24.5	27.4	17.3	9.2	2.3	57.3
	23103					0.9	3.0	11.4	23.5	36.2	23.3	1.7		57.4
	23174				0.5	1.4	6.2	16.1	33.4	31.3	10.9	0.6		57.2
	23204					1.4	4.7	14.5	29.0	32.9	14.1	3.6		57.0

TABLE 3. Grouping by average total number of carbon atoms in mycolic acids

Species	No. of strains	Strains with the following avg total no. of carbon atoms in mycolic acids:						
		~50	52	53	54	55	56	57
<i>N. asteroides</i>	18	23019, 23099	23094, 23173	23046, 23064, 23169, 23175, 23183, 23189, 23203	23008, 23049, 23182, 23206 ^T	23170, 23172, 23205		
<i>N. farcinica</i>	17		23013, 23101, 23166	23004, 23087, 23102, 23157 ^T , 23162, 23163, 23164, 23165, 23167, 23168, 23201	23055, 23171, E12618			
<i>N. nova</i>	17					23038, 23044	23006, 23032, 23088, 23096, 23177, 23181, 23210	23012, 23095 ^T , 23103, 23174, 23192, 23196, 23204, 23209

both *N. asteroides* and *N. farcinica* had C₅₄ mycolic acids with C_{16:0} α-chains as the most abundant molecular species (range, C₄₄ to C₅₈ with zero to three double bonds).

Seventeen strains of *N. nova* had C₅₆ or C₅₈ mycolic acids (total carbon numbers) with C_{16:0}, C_{18:0}, or C_{18:1} α-chains (range, C₅₀ to C₆₂ with zero to four double bonds). The mycolic acid composition of *N. nova* was clearly different from the mycolic acid compositions of the other two species. Consequently, our data revealed that *N. nova* is a good species that can be distinguished from *N. asteroides* and *N. farcinica*. This finding was supported by the results of other studies, such as DNA complementarity (31), 16S rRNA (6, 16), antibiotic sensitivity (28), opacification of Middlebrook agar (8), and detection of preformed enzyme (5) studies.

GC-MS analysis was very effective because it gave precise information concerning the structure of the α-branch and straight alkyl chain unit of mycolic acids with a small amount of sample. The grouping by average total carbon numbers of mycolic acid coincided well with the results of a DNA hybridization study. Biosynthesis of mycolic acids is thought to include many enzymatic steps that are performed by complex multienzyme systems (15, 23, 24). Therefore, we suppose that the mycolic acid structure is determined by the bulk of genes. A taxonomy based on mycolic acid composition would be more consistent with a taxonomy based on genetic characteristics than with a taxonomy based on numerical phenetic characteristics. Mycolic acid analysis by GC-MS combined with DNA or RNA analysis appears to be sufficient for definite identification of *Nocardia* strains and can be recommended for routine use.

We are trying to separate molecular species with odd or even numbers of total carbons and double bonds by capillary GC-MS and expect to be able to differentiate *N. asteroides* and *N. farcinica*. A comprehensive analysis of all *Nocardia* species will be reported in a later paper.

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