JOURNAL OF BACTERIOLOGY, Aug. 1982, p. 828–837
0021-9193/82/080828-10$02.00/0

Vol. 151, No. 2

Mycolic Acid Composition and Thermally Adaptative Changes in *Nocardia asteroides*

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Received 25 January 1982/Accepted 29 March 1982

The nocardomycolic acid compositions of extractable and the cell wall-bound lipids from five strains of *Nocardia asteroides* (A-23007, A-23094, B-23006, B-23095, and IFO 3384) were compared by using gas chromatography-mass spectrometry. The molecular species composition of mycolic acid differed significantly among the strains of *N. asteroides*. The A-23007 strain possessed the shortest species, centering at $C_{44(46)}$, and the A-23094 and IFO-3384 strains followed, each centering at C_{52}. The B-23006 and B-23095 strains possessed the longest species, centering at C_{56} or C_{54}, thus indicating that *N. asteroides* strains accommodate a heterogeneous group in respect to carbon numbers of mycolic acids. The double-bond isomers of mycolic acids from the representative strain IFO 3384 were fully separated and analyzed by argentation thin-layer chromatography, followed by gas chromatography-mass spectrometry. The reference strain (IFO 3384) possessed up to four double bonds on the straight chain of mycolic acids ranging from C_{46} to C_{60}. All of the species possessed a C_{14} alkyl branch at C-2. The more highly unsaturated subclasses consisted of the longer-chain mycolic acids. Marked changes in mycolic acid composition were induced by altering the growth temperature of strain IFO 3384. The cells grown at the higher temperature (50°C) contained more saturated mycolic acids, whereas those grown at the lower temperature (17°C) had more polyunsaturated (up to tetraenoic) mycolic acids, although a significant difference in carbon chain length was not detected. These changes in the degree of unsaturation of mycolic acids occurred shortly after shifting the growth temperature from 17 to 50°C at logarithmic stages of the bacterial growth, thus indicating that *N. asteroides* can adapt to changes in the environmental temperature by altering the structure of mycolic acids of the cell walls.

Mycolic acids (α-alkyl and β-hydroxy very long-chain fatty acids) are the most characteristic cell wall components in bacteria belonging to the order *Actinomycetales* and contribute to the physiological properties of cell walls such as acid fastness and hydrophobicity. The structure of mycolic acids varies by the families or species; mycobacteria generally possess C_{60} to C_{80} acids (3, 11, 15, 16, 22), whereas nocardia and rhodococci possess shorter-chain homologs such as C_{40} to C_{60} acids (5, 8, 13, 17).

Recently, using gas chromatography-mass spectrometry (GS/MS), the mycolic acid compositions of 5 *Nocardia* species ("*Nocardia* sensu stricto") and 11 *Rhodococcus* clusters were examined in detail, and each species was shown to have characteristic profiles of mycolic acid composition (4, 28, 29), although the total number of carbon atoms covered a wide range from C_{28} to C_{68} among the above taxa. Thus, the structure of mycolic acid seems to be determined genetically, which is useful for chemotaxonomy or identification of isolated strains. On the other hand, more recently we have also reported that the mycolic acid compositions of nocardia and mycobacteria vary significantly with the environmental conditions such as growth temperature (23, 25).

In *Nocardia rubra*, cells grown at higher temperature contain more saturated mycolic acid, whereas cells grown at lower temperature possess more dienoic acids (23).

Nocardia asteroides strains have more highly unsaturated mycolic acids (up to tetraenoic) (28, 29). To establish taxonomical homogeneity and to generalize the adaptative changes in mycolic acid composition, I compared the molecular species of five *N. asteroides* strains and the temperature-induced changes in mycolic acids.

The present paper demonstrates that strains of

N. asteroides are considerably heterogeneous as has been shown morphologically (9, 26). Furthermore, I report that the mycolic acid composition varies with the environmental temperature over wide ranges of unsaturation.

MATERIALS AND METHODS

The strains of *N. asteroides* used for analysis were IFO 3384, 23006, 23007, 23094, and 23095. The latter four strains were supplied by M. Tsukamura, National Chubu Chest Hospital, Obu, Japan. Cells were grown at various temperatures ranging from 15 to 50°C for 48 to 72 h on a rotary shaker in a medium containing 1% glucose, 0.5% peptone, and 0.2% yeast extract, with the final pH adjusted to 7.0. The cells were harvested by centrifugation at the early stationary phase of growth. The lipids were extracted three times with a mixture of chloroform and methanol (2:1, vol/vol). The fatty acids were obtained by hydrolysis with 10% KOH in methanol of the extractable lipids and the residual cell wall-bound lipids. After acidification with 6 N HCl, fatty acids were extracted with *n*-hexane and transmethylated with benzene–methanol–H_2SO_4 (10:20:1, vol/vol) for 1.5 h under reflux. The resultant methyl esters were extracted with *n*-hexane and separated on a thin-layer plate of Silica Gel G (Merck) with a solvent system of *n*-hexane–diethyl ether (4:1, vol/vol). After the mycolic acid esters were eluted from the thin-layer plate with chloroform, they were further separated on 10% silver nitrate-impregnated silica gel according to the number of double bonds. The saturated, monoenoic, dienoic, trienoic, and tetraenoic mycolic acid esters were well separated with a solvent system of chloroform–methanol (80:1.5, vol/vol). The subclasses of mycolic acid esters were located by spraying with 0.5% rhodamine 6-G in methanol and then recovered from the gel with diethyl ether. The resultant mycolic acid methyl esters were trimethylsilylated with bis-trimethylsilyltrifluoroacetamide in pyridine as described previously (28, 29).

The trimethylsilylated derivatives of the mycolic acid were injected into a GC/MS apparatus (Hitachi M-60) equipped with a glass column (0.5 m by 3 mm) that was packed with Diasolid ZT. The columns were maintained at 320°C, and both the separator and the injector were kept at 350°C. The ionization energy was 20 eV, and the accelerating voltage was 3.2 kV. The mass spectra of trimethylsilylated methyl mycolate were recorded at the top of gas chromatographic peaks, and the mycolic acid composition was calculated from peak area percentages. For the simple gas chromatographic separation of mycolic acids, a Shimazu GC-4BF apparatus equipped with flame ionization detector was operated at 250 to 350°C (temperature programmed at 3°C/min). For the mass fragmentographic analysis, a certain acceleration voltage was maintained precisely by a microcomputer system, and the relative abundances of mycolic acids were quantitated from the fragmentograms of [M-15], which is derived from loss of a methyl group from molecular ions. The mass fragmentographic analysis enabled us to estimate individual molecular species differing in double-bond numbers in a specific carbon-numbered molecule, without argentation thin-layer chromatography. The solvents used for the above experiments were redistilled before use.

RESULTS

Thin-layer and gas chromatographic separations of mycolic acids. Thin-layer chromatograms of fatty acid methyl esters of the extractable and cell wall-bound lipids from four strains of *N. asteroides* are shown in Fig. 1. The total fatty acid methyl esters gave two spots: the upper spot corresponded to nonpolar fatty acid methyl esters, and the lower spot corresponded to mycolic acid methyl esters possessing R_f values of 0.35 to 0.45. The R_f values of mycolic acid esters obtained from five strains differed slightly from each other, indicating that the chain length of each mycolic acid varied according to the strain of *N. asteroides*. After thin-layer chromatographic separation, mycolic acid methyl esters were isolated, trimethylsilylated, and subjected to GC/MS analysis.

The mycolic acid compositions of the extractable and cell wall-bound lipid fractions are shown in Table 1. It was noted that the mycolic acid compositions of five strains of *N. asteroides* differed significantly. Strain 23007 possessed a C_{36} to C_{50} mycolic acid centering at C_{44} or C_{46}; strains 23094 and IFO 3384 had a C_{44} to C_{58} mycolic acid centering at C_{52}; and strains 23006 and 23095 had a C_{48} to C_{60} mycolic acid centering at C_{54} or C_{56}.

Argentation thin-layer chromatograms of nocardomycolic acid methyl esters of *N. asteroides* IFO 3384 showed five spots, corresponding to saturated, monoenoic, dienoic, trienoic, and tetraenoic acid esters. Each subclass of mycolic acid methyl ester separated by argentation thin layer chromatography was recovered

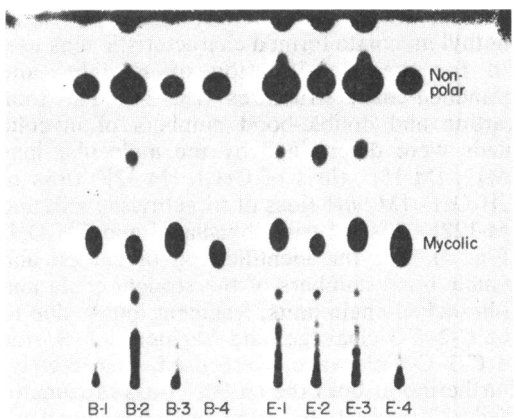

FIG. 1. Thin-layer chromatograms of fatty acid methyl esters from the extractable (E) and cell wall-bound (B) lipids of *N. asteroides* strains. Nonpolar, Nonpolar fatty acid methyl esters; Mycolic, nocardomycolic acid methyl esters. E-1 and B-1, strain 23095; E-2 and B-2, strain 23094; E-3 and B-3, strain 23006; E-4 and B-4, strain 23007. The plate was developed with a solvent of *n*-hexane–diethyl ether (4:1, vol/vol).

TABLE 1. Mycolic acid composition of extractable and cell wall-bound lipids in five strains of *N. asteroides*

Mycolic acid	% Composition in lipid of following strain:								Total (strain 3384)
	Extractable				Cell wall bound				
	23007	23094	23006	23095	23007	23094	23006	23095	
C_{36}	1.3				1.1				
C_{38}	4.4				4.2				
C_{40}	10.2				9.4				
C_{42}	20.5				15.7				
C_{44}	27.4	6.5			29.0	2.7			tr
C_{46}	21.8	5.7			30.9	4.9			1.7
C_{48}	14.4	12.2		0.6	9.7	13.1		1.5	5.7
C_{50}	tr	24.5	0.7	3.9	tr	25.9	2.4	4.0	19.4
C_{52}		27.6	4.3	21.1		34.2	6.9	18.1	44.6
C_{54}		10.6	12.8	45.1		18.0	29.2	30.5	16.8
C_{56}		12.9	36.1	25.6		1.2	41.8	26.7	7.8
C_{58}		tr	35.6	3.7		tr	18.2	11.6	tr
C_{60}			10.5	tr			1.5	1.6	

from thin-layer plates and further analyzed by gas chromatography as their trimethylsilylated ether derivatives (Fig. 2). The gas chromatographic patterns of mycolic acid molecular species of each subclass, which differed in the degree of unsaturation, resembled each other, ranging from C_{48} to C_{64}. The most abundant species, however, varied, with C_{54} predominating in saturated, monoenoic, and dienoic subclasses. On the other hand, the trienoic mycolic acid contained mostly C_{56} or C_{58}, and tetraenoic mycolic acid contained mostly C_{60}.

Mass spectrometric analysis of mycolic acids. The mass spectra of individual trimethylsilylated methyl mycolates isolated by argentation thin-layer chromatography and GC/MS were recorded, and those of C_{54} mycolic acid (saturated, monoenoic, dienoic, trienoic, and tetraenoic) are shown in Fig. 3. As shown in previous reports, the fragmentation of trimethylsilylated methyl mycolate formed characteristic ions useful for the identification of straight- and branched-chain structures (28, 29). The total carbon and double-bond numbers of mycolic acids were determined by the molecular ions $[M]^+$, $[M-15]^+$ (loss of CH_3), $[M-32]^+$ (loss of CH_3OH), $[M-90]^+$ (loss of trimethylsilanol), and $[M-122]^+$ (loss of trimethylsilanol and CH_3OH) (Fig. 4). For the identification of carbon and double-bond numbers of the straight-chain and α-branched-chain units, fragment ion A, due to the C-2–C-3 cleavage, and fragment ion B, due to C-3–C-4 cleavage, were used, respectively. Furthermore, both the $[A-90]^+$ (loss of trimethylsilanol) and $[B-29]^+$ (loss of CHO) ions were also useful for the determination of mycolic acid structure. These results are summarized in Table 2. After argentation thin-layer chromatography, the single molecular ion $[M]^+$ of each gas chromatographic peak was clearly demonstrated. In *N. asteroides*, the straight-chain unit varied from C_{32} to C_{48} according to the total number of carbon atoms, and up to four double bonds were located generally in the straight-chain alkyl unit. On the other hand, a C_{14} alkyl chain (saturated, or monoenoic in small amounts) commonly existed at C-2. The molecular species composition in each subclass (satu-

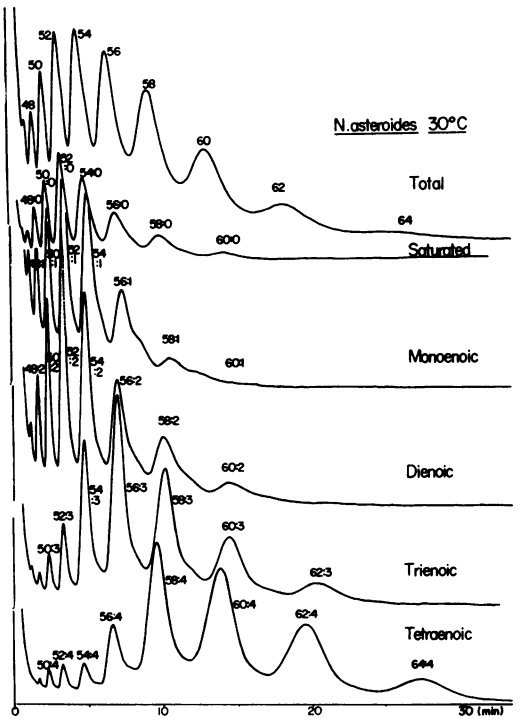

FIG. 2. Gas chromatograms of nocardomycolic acid subclasses (saturated, monoenoic, dienoic, trienoic, and tetraenoic acids) as trimethylsilylated ether derivatives of methyl esters from *N. asteroides* IFO 3384. The gas chromatographic analysis was performed with a Shimazu GC-6A apparatus equipped with flame ionization detector. The glass column (3 mm by 0.5 m) was maintained at 320°C, and the injector was at 375°C.

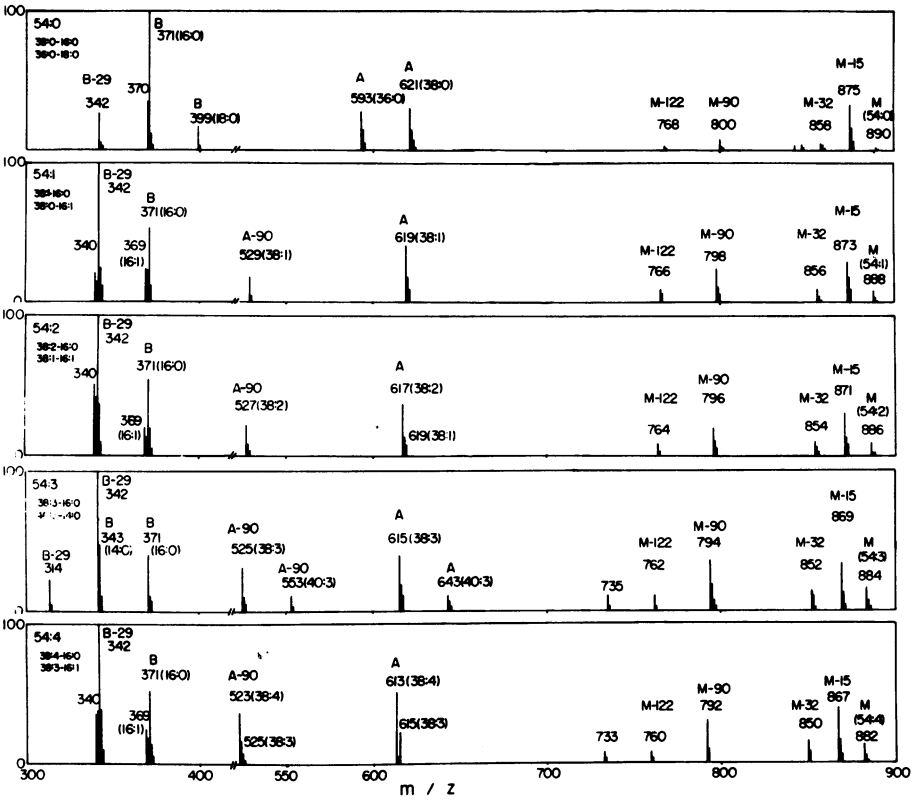

FIG. 3. Mass spectra of fully separated trimethylsilylated methyl mycolates from *N. asteroides* IFO 3384. From top to bottom: C_{54} saturated, C_{54} monoenoic, C_{54} dienoic, C_{54} trienoic, and C_{54} tetraenoic acids. Mass spectra were recorded with a Hitachi M-60 GC/MS system. The conditions are described in the text.

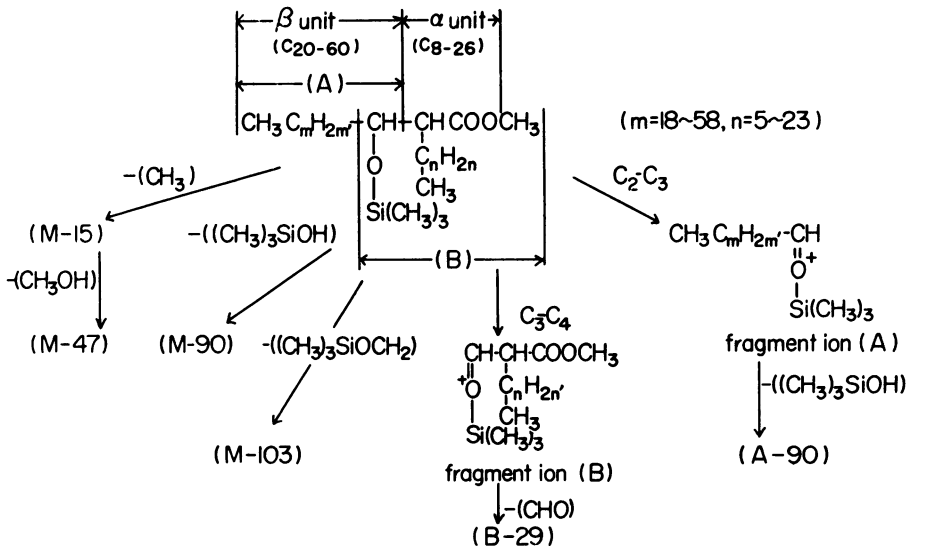

FIG. 4. General structure and mass fragmentation pattern of trimethylsilyl derivatives of methyl mycolate.

TABLE 2. GC/MS analysis of trimethylsilylated methylmycolates from *N. asteroides* IFO 3384[a]

Mycolic acid subclass	No. of carbon atoms:no. of double bonds	% of total	m/z			Straight chain (β unit)	Branched chain (α unit)
			[M+]	Fragment ion			
				A	B		
Saturated	48:0	5.7	806	537	371	32:0	16:0
	50:0	10.9	834	565	371	34:0	16:0
				(593)	(343)	(36:0)	(14:0)
	52:0	20.1	862	593	371	36:0	16:0
				(621)	(343)	(38:0)	(14:0)
	54:0	24.6	890	621	371	38:0	16:0
				(593)	(399)	(36:0)	(18:0)
	56:0	21.7	918	649	371	40:0	16:0
				(621)	(399)	(38:0)	(18:0)
	58:0	12.2	946	677	371	42:0	16:0
	60:0	4.8	974	705	371	44:0	16:0
Monoenoic	48:1	8.2	804	535	371	32:1	16:0
	50:1	13.3	832	563	371	34:1	16:0
				(565)	(369)	(34:0)	(16:1)
	52:1	21.8	860	591	371	36:1	16:0
				(593)	(369)	(36:0)	(16:1)
	54:1	28.3	888	619	371	38:1	16:0
				(621)	(369)	(38:0)	(16:1)
	56:1	19.7	916	647	371	40:1	16:0
	58:1	8.7	944	675	371	42:1	16:0
	60:1	tr	972	703	371	44:1	16:0
Dienoic	48:2	6.5	802	533	371	32:2	16:0
	50:2	10.3	830	561	371	34:2	16:0
				(563)	(369)	(34:1)	(16:1)
	52:2	17.1	858	589	371	36:2	16:0
				(591)	(369)	(36:1)	(16:1)
	54:2	21.1	886	617	371	38:2	16:0
				(619)	(369)	(38:1)	(16:1)
	56:2	17.1	914	645	371	40:2	16:0
	58:2	16.6	942	673	371	42:2	16:0
	60:2	11.2	970	701	371	44:2	16:0
Trienoic	50:3	2.6	828	559	371	34:3	16:0
				(587)	(343)	(36:3)	(14:0)
	52:3	5.1	856	587	371	36:3	16:0
				(615)	(343)	(38:3)	(14:0)
	54:3	14.3	884	615	371	38:3	16:0
				(643)	(343)	(40:3)	(14:0)
	56:3	25.9	912	643	371	40:3	16:0
				(645)	(369)	(40:2)	(16:1)
	58:3	25.3	940	671	371	42:3	16:0
	60:3	18.6	968	699	371	44:3	16:0
	62:3	8.2	996	727	371	46:3	16:0
Tetraenoic	50:4	1.2	826	557	371	34:3	16:0
	52:4	2.1	854	585	371	36:4	16:0
				(587)	(369)	(36:3)	(16:1)
	54:4	2.9	882	613	371	38:4	16:0
				(615)	(369)	(38:3)	(16:1)
	56:4	8.2	910	641	371	40:4	16:0
				(643)	(369)	(40:3)	(16:1)
	58:4	23.5	938	669	371	42:4	16:0
				(671)	(369)	(42:3)	(16:1)
	60:4	30.4	966	697	371	44:4	16:0
	62:4	22.9	994	725	371	46:4	16:0
	64:4	8.8	1022	753	371	48:4	16:0

[a] Fragment ion and corresponding alkyl chain in small amounts are shown within parentheses.

rated, monoenoic, dienoic, trienoic, and tetraenoic) was calculated (Table 2).

Mass fragmentographic determination. Since the nocardomycolic acid contained several molecular species in a subclass differing in double-bond numbers, the amounts of individual components were determined by mass fragmentography by monitoring $[M-15]^+$ ions. Figure 5 shows mass fragmentograms of trimethylsilylated derivatives of total mycolic acid methyl esters from the cells grown at low (17°C) and high (50°C) temperatures. By monitoring $[M-15]^+$ ions of trimethylsilylated derivatives of saturated, monoenoic, dienoic, trienoic, and tetraenoic methyl mycolates, using the acceleration voltage alternation system of the mass spectroscope, all of the single molecular species differing in carbon and double-bond numbers were estimated quantitatively without separation by argentation thin-layer chromatography. As an example, for the saturated mycolic acids from the cells grown at 17°C, [M-15] ions with *m/z* values of 763, 791, 819, 847, 875, 903, 931, and 959, each corresponding to $C_{46:0}$, $C_{48:0}$, $C_{50:0}$, $C_{52:0}$, $C_{54:0}$, and $C_{56:0}$, respectively, were monitored. The peak area percentages were calculated, and the resultant mycolic acid compositions of high- and low-temperature-grown cells are summarized in Table 3. It was of great interest that the ratio of saturated, monoenoic, dienoic, trienoic, and tetraenoic mycolic acids varied dramatically by the growth temperature. The most abundant mycolic acid in the cells grown at 17°C was C_{50} dienoic, whereas those in the cells grown at 50°C were C_{52} and C_{54} saturated acids. Although the carbon numbers of mycolic acid did not change significantly as the growth temperature was varied, the composition of double-bond isomers varied remarkably. The amounts of total saturated mycolic acids from the cells grown at 17 and 50°C were 7.0 and 57.0%, respectively. The amounts of total polyunsaturated mycolic acids (total of dienoic, trienoic, and tetraenoic) from the cells grown at 17 and 50°C were 78.4 and 11.5%, respectively.

Changes in mycolic acid composition. Changes in the composition of double-bond isomers in selected carbon-numbered mycolic acids by the growth temperature are shown in Fig. 6. As the cultivation temperature was elevated from 17 to 50°C, the saturated acids increased, commonly with a concomitant decrease in polyenoic acids (dienoic, trienoic, and tetraenoic acids) throughout C_{50} to C_{56} mycolic acids. The monoenoic acids generally predominated in the cells grown at 30°C. The tetraenoic acids could be hardly detected in cells grown at 50°C, whereas the trienoic and tetraenoic acids existed abundantly in cells grown at 17°C.

Since the degree of unsaturation of nocardomycolic acid changed markedly upon the adaptative response to growth temperature, we ex-

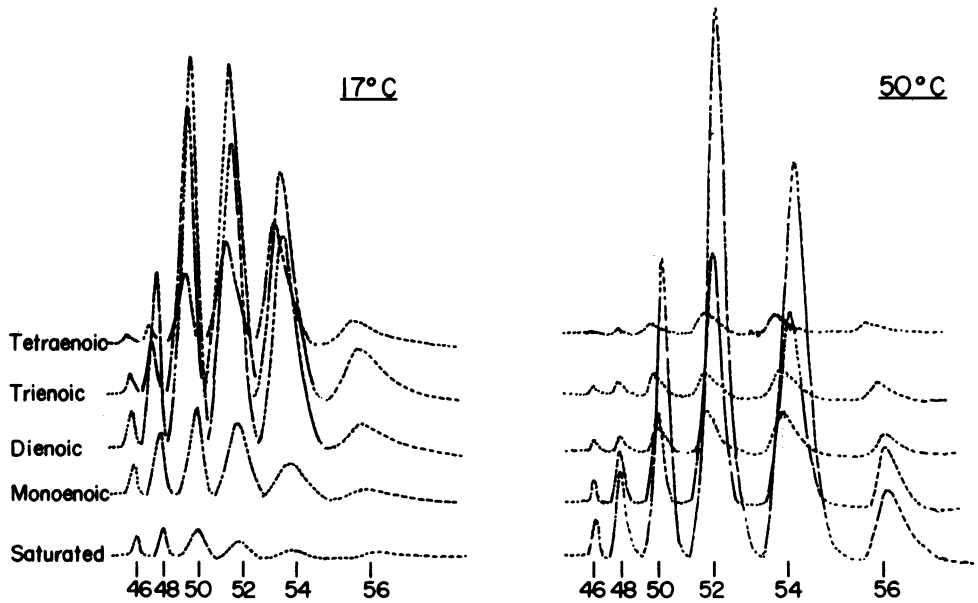

FIG. 5. Mass fragmentograms of trimethylsilylated methyl mycolate from *N. asteroides* IFO 3384 grown at 17 or 50°C. Mass fragmentographic analysis was performed by monitoring [M-15] ions characteristic for each carbon-numbered, saturated, monoenoic, dienoic, trienoic, and tetraenoic molecular species. For the saturated subclass, as an example, *m/z* values of 763 ($C_{46:0}$), 791 ($C_{48:0}$), 819 ($C_{50:0}$), 847 ($C_{52:0}$), 875 ($C_{54:0}$), and 903 ($C_{56:0}$) were monitored with a multiple ion-detector system.

TABLE 3. Mycolic acid composition of *N. asteroides* IFO 3384 grown at high (50°C) and low (17°C) temperatures

Mycolic acid	% Composition									
	17°C-Grown cells					50°C-Grown cells				
	Saturated	Monoenoic	Dienoic	Trienoic	Tetraenoic	Saturated	Monoenoic	Dienoic	Trienoic	Tetraenoic
C_{46}	0.9	1.5	1.5	0.8	0.4	0.7	0.7	0.1	0.1	
C_{48}	1.7	2.7	8.3	2.0	0.8	3.4	2.4	0.3	0.2	0.1
C_{50}	2.3	4.9	14.5	8.0	1.5	8.4	3.2	0.5	0.4	0.2
C_{52}	1.6	3.5	11.2	10.5	2.5	20.3	11.4	2.4	0.6	0.5
C_{54}	0.5	1.7	6.0	6.2	1.0	20.3	10.0	2.6	0.8	0.3
C_{56}		0.3	0.9	1.8	0.5	3.9	3.7	1.6	0.6	0.2
Total	7.0	14.6	42.4	29.3	6.7	57.0	31.4	7.5	2.7	1.3

amined the changes in these types of fatty acids shortly after shifting up the growth temperature from 17 to 50°C at the logarithmic phase of growth. A significant increase in saturated mycolic acids and a concomitant decrease in polyunsaturated mycolic acids (dienoic, trienoic, and tetraenoic acids) were already observed at 2 h after shifting up the cultivation temperature (Fig. 7). After 20 h, the saturated mycolic acids predominated in C_{46}, C_{48}, C_{50}, C_{52}, C_{54}, and C_{56} molecules, commonly with a concomitant decrease in dienoic, trienoic, and tetraenoic mycolic acids. Therefore, from the results obtained above, it was revealed that the *N. asteroides* can synthesize specific carbon- and double-bond-numbered mycolic acids under specific environmental conditions such as growth temperature.

DISCUSSION

Lipid analyses are especially useful in the classification and identification of actinomycetes (1, 10, 14, 20, 21). Much attention has been paid to the analysis of mycolic acids, which are associated mostly with the cell walls of acid-fast bacteria. Mycolic acids are high-molecular-weight β-hydroxy fatty acids with a long chain in the α position, and they generally exist as mixtures of homologs (4, 24, 28, 29). Nocardomycolic acids have already been reported to have a chain length of about C_{50} (8, 13), which is different from those of mycolic acids from mycobacteria and corynebacteria. Therefore, finding differences in the structure of the respective methyl mycolates provides a reliable way of distinguishing between corynebacteria, mycobacteria, and nocardia (20, 21, 24). Recently, mycolic acids of C_{38} to C_{46} have been found in *Nocardia corallina* (4) and *Nocardia rhodochrous* (18), and Yano et al. reported that *Nocardia erythropolis* has mycolic acids with a wide variety of chain lengths, ranging from C_{32} to C_{48} (28). In subsequent studies with GC/MS, Yano et al. also investigated the mycolic acid composition of 11 strains of *Rhodococcus* and five

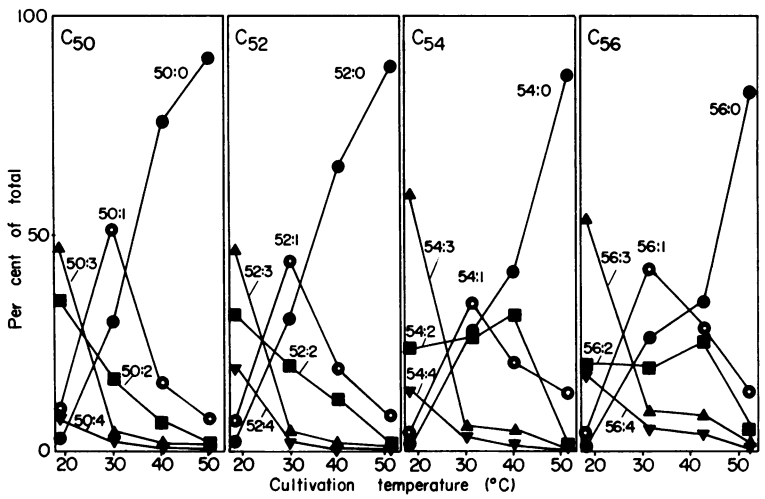

FIG. 6. Changes in mycolic acid subclass composition by cultivation temperature in *N. asteroides* IFO 3384. Symbols: ●, saturated; ○, monoenoic; ■, dienoic; ▲, trienoic; ▼, tetraenoic.

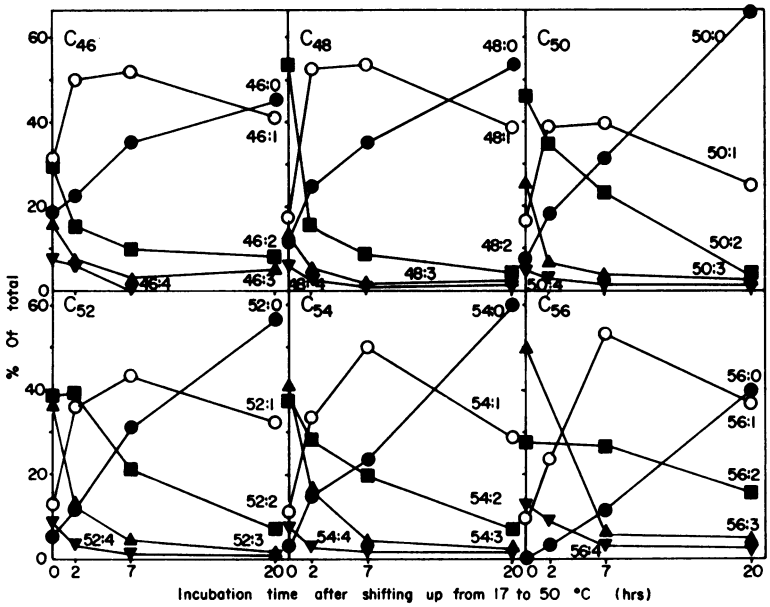

FIG. 7. Changes in mycolic acid composition of *N. asteroides* IFO 3384 after shifting up the cultivation temperature. *N. asteroides* was grown at 17°C until mid-log stage, and then the incubation temperature was elevated to 50°C. Cells were harvested and analyzed at appropriate intervals (0, 2, 7, and 20 h). Symbols: ●, saturated; ○, monoenoic; ■, dienoic; ▲, trienoic; ▼, tetraenoic.

strains of *Nocardia* sensu stricto (true nocardia) and demonstrated the wide distribution of molecular species, ranging from C_{28} to C_{68}, among these taxa (29). From these reports, the *Rhodococcus* group has been shown to be extremely heterogeneous, whereas true nocardia (*Nocardia* sensu stricto) are recognized as homogeneous. However, in the present study, the five strains of *N. asteroides* showed slightly different R_f values of methyl mycolate on thin-layer plates, suggesting that each strain possesses different molecular species as has been already reported (2, 19). GC/MS analysis clearly revealed further differences in mycolic acid composition. Based on the mycolic acid composition, the five strains of *N. asteroides* examined above were classified into three types, the first ranging from C_{36} to C_{50} and centering at C_{44} for strain 23007, the second ranging from C_{44} to C_{58} and centering at C_{52} for strains IFO 3384 and 23094, and the third ranging from C_{48} to C_{60} and centering at C_{54} or C_{56} for strains 23006 and 23095. The double-bond isomer composition resembled roughly also those of *N. asteroides* reported previously (5, 8, 9). However, the four strains IFO 3384, 23094, 23006, and 23095 possessed up to tetraenoic acids, and this is the first detailed report that *N. asteroides* can synthesize tetraenoic mycolic acids. Therefore, *N. asteroides* is assumed to be heterogeneous in respect to chemotaxonomy. Since Tsukamura (26) and Goodfellow (9) already reported that *N. asteroides* strains are morphologically heterogeneous, it may not be surprising that chemical differences between strains exist and that this species may be classified further into two or three subspecies.

In *N. asteroides*, the occurrence of trehalose mono- and dimycolates has been demonstrated (27), and the different mycolic acid compositions among lipid classes in *N. asteroides* also have been reported (12). To find out the lipid specificity in mycolic acid composition, I compared those in both the extractable and the cell wall-bound lipid fractions. Slight differences were observed commonly in the five strains of *N. asteroides*. However, under these conditions of bacterial growth (harvested at the early stationary phases), no significant differences between both lipid classes were detected. This may be due to differences in the strains of *N. asteroides* used for analysis.

The most characteristic feature of nocardomycolic acids is the abundant occurrence of polyunsaturated acids. However, certain carbon-numbered isomers possessing different double-bond numbers cannot be separated by gas chromatography. Argentation thin-layer chromatography gave a good separation of the subclasses differing in double-bond numbers. However, again, the accurate estimation of double-bond isomers is extremely difficult since the recovery of the fatty acid methyl esters from the silver nitrate-impregnated thin-layer plate is not

necessarily quantitative. I have introduced a mass fragmentographic analysis of trimethylsilylated methyl mycolate for the determination of double-bond isomers by monitoring different mass fragment ions such as $[M-15]^+$ or $[M-90]^+$ for the total mycolic acid molecule, fragment ion A for the straight chain, or fragment ion B for the α branch (29). This method gave good results for the quantitative analysis of double-bond isomers such as saturated, monoenoic, dienoic, trienoic, and tetraenoic nocardomycolic acids without any separation by argentation thin-layer chromatography. By using above method, the relative concentration of all of the molecular species was determined quantitatively.

It was noted that in *N. asteroides*, marked changes in mycolic acid composition were observed by varying the growth temperature; the cells grown at 50°C consisted of saturated acid almost completely, whereas those grown at 17°C possessed dienoic, trienoic, and tetraenoic acids abundantly. Since the generation time for *N. asteroides* IFO 3384 at 50°C were 4 h and the changes were observed already at 2 h after shifting of growth temperature, it was suggested the adaptative synthesis (not the alteration of existing lipid molecules) of mycolic acid occurred very quickly (in at least one doubling time). As we have reported on the similar changes in *N. rubra* previously (23), these types of adaptative variation seem to be specific for and common among the true nocardia and their related group. *Mycobacterium phlei* differed from nocardia in that the temperature-induced changes in the mycolic acid molecule occurred at the alkyl chain length. Cells grown at lower temperature contained shorter homologs, and those grown at higher temperature possessed longer homologs (25). These changes in the degree of unsaturation or the chain length of mycolic acid by the cultivation temperature seemed undoubtedly to be related to the rigidity of cell walls as in the cases of temperature-induced changes in fatty acid composition of membrane phospholipids (6, 7). Since *Nocardia* and related *Rhodococcus* species were shown to possess a great variety of mycolic acids, and their compositions were demonstrated to vary significantly by the growth temperature, it would be of particular interest to know the regulatory mechanism(s) in mycolic acid synthesis, genetically and environmentally. For the definitive elucidation of regulation in the cell wall mycolic acid biosynthesis, I am now examining the incorporation of radiolabeled and stable isotope-labeled precursors in vivo and in vitro.

ACKNOWLEDGMENTS

I thank M. Tsukamura for supplying strains of *N. asteroides*, I. Yano for valuable discussions and suggestions, and K. Mino for preparation of this manuscript.

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