Occurrence of Corynomycolic Acids in Strains of *Nocardia otitidis-caviarum*

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Studies of the hydroxylated fatty acids in strains of *Nocardia otitidis-caviarum* showed variations in mycolic acid patterns, some strains containing only nocardomycolic acids and other strains having both nocardomycolic and corynomycolic acids. Mass spectroscopy showed that most of the nocardomycolic acids contained 52 to 56 carbon atoms and two double bonds in the main chain; the corynomycolic acids had saturated or monounsaturated chains and 30 to 34 carbon atoms. In the strains having both types of mycolic acid, only nocardomycolic acids were found as cell wall components.

INTRODUCTION

In the corynebacteria, mycobacteria, nocardiae and rhodococci group several chemical markers have been proposed (Minnikin & Goodfellow, 1980) but mycolic acid patterns remain the most widely used. Mycolic acids are high molecular weight 3-hydroxy 2-alkyl fatty acids of variable length: C₆₀ to C₉₀ for mycolic acids of mycobacteria with oxygen functions in the main chain, C₅₀ to C₅₆ for nocardomycolic acids with saturated or unsaturated main chains, and C₃₀ to C₃₆ for corynomycolic acids of corynebacteria.

Ioneda *et al.* (1970), however, showed the presence of trehalose dicorynomycolates in a strain of *Nocardia asteroides*. This finding seemed to be exceptional and indeed the simultaneous presence of two types of mycolic acid has never been reported since. In a systematic investigation of the lipids from several strains of *Nocardia otitidis-caviarum*, to determine the distribution of a specific glycolipid (Pommier & Michel, 1985), mycolic acids were isolated and studied by mass spectrometry. This study reports that, in some strains, corynomycolic acids were found in addition to nocardomycolic acids.

METHODS

Strains and cultivation. The strains of *Nocardia* are indicated in Table 1. They were grown in Roux bottles in Sauton's medium (Sauton, 1912) at 37 °C for 3 weeks, harvested by filtration, washed with distilled water and freeze-dried.

Extraction of lipids and fatty acids. Lipids were extracted three times from dried cells (0.5 g) with 100 ml chloroform/methanol (2:1, v/v). The crude extract was washed according to Folch *et al.* (1957). Neutral lipids were eluted by chloroform from a silicic acid column and fatty acids were obtained by hydrolysis of neutral lipids with 2.5 M-methanolic KOH. Methyl esters were prepared from fatty acids by treatment with diazomethane.

Hydroxylated and non-hydroxylated fatty acid methyl esters were separated by thin-layer chromatography (TLC) on silica gel G with hexane/diethyl ether (4:1, v/v). Methyl esters were visualized by spraying with a 0.01 % ethanolic rhodamine B solution or using a bromothymol blue/sodium hydroxide reagent (Michalec *et al.*, 1975). Hydroxylated fatty acids were obtained by preparative TLC.

Analysis of cell wall fatty acids. Cell walls were obtained by sonic disruption of defatted bacteria. The disruption was checked by microscopy. The crude cell walls were hydrolysed with methanolic KOH and fatty acids obtained after acidification by extraction with diethyl ether, were esterified with diazomethane and purified by preparative TLC.

Gas chromatography/mass spectrometry (GC/MS) analysis. The trimethylsilyl ether derivatives of hydroxylated fatty acid methyl esters were prepared by the method of Tomiyasu *et al.* (1981) and were separated on an Intersmat

Table 1. *Designation and sources of bacterial strains*

Strain no.	Designation and source
IM 1379, IM 1380, IM 1381	<i>N. otitidis-caviarum</i> . Institut Mérieux, Laboratoire de Marcy l'Etoile, 69752 Charbonnières-les-Bains, France
IMRU 1259, IMRU 1355	<i>N. otitidis-caviarum</i> . H. Lechevalier, Rutgers University, New Brunswick, NJ, USA
R416, R1316	<i>N. otitidis-caviarum</i> . M. Tsukamura, The National Chubu Hospital, Obu Aichi 474, Japan
N36, N314, N939, N940, N942, N943, N945	<i>N. otitidis-caviarum</i> . M. Goodfellow, Department of Microbiology, The University, Newcastle upon Tyne NE1 7RU, UK
N6*, N5*	<i>N. asteroides</i> . M. Ridell, Institute of Medical Microbiology, University of Göteborg, Guldhedsgatan 10, S413 46 Göteborg, Sweden

* These strains contained a glycolipid characteristic of *N. otitidis-caviarum* and should be included in the *N. otitidis-caviarum* species (Pommier & Michel, 1985).

chromatograph equipped with a stainless steel column (0.3 × 150 cm) packed with 10% Silicone SE 30 on Chromosorb PAW (80–100 mesh). The column was maintained at 300 °C for the analysis of the trimethylsilyl ether derivatives and at 180 °C for GC of mycolic methyl esters (Etémadi, 1967).

Electron impact (70 eV) mass spectra of methyl mycolates were determined on an AEI MS 902 instrument and isobutane chemical ionization spectra were determined on a VG ZAB HF instrument.

RESULTS

Identification of mycolic acids

Among the strains of *Nocardia otitidis-caviarum* which had been studied for their glycolipid composition (Pommier & Michel, 1985) two strains, R1316 and R416, were examined more extensively for their lipid components. TLC of total fatty acid methyl esters showed three spots, A, B and C (Fig. 1). Spot A (R_F 0.82) contained a mixture of non-hydroxylated methyl esters consisting of $C_{16:0}$, $C_{18:0}$, $C_{18:1}$ and $C_{19:br}$ (tuberculostearic) fatty acids, as shown by GC.

The components of spot B (R_F 0.44) migrated with nocardomycolic methyl esters isolated from various *Nocardia* strains (Maurice *et al.*, 1971) and they accounted for 16% (strain R416) and 28% (strain R1316) of the total fatty acid methyl esters. The structure of these nocardomycolic methyl esters was determined by MS. They gave a characteristic fragmentation pattern similar to those of nocardomycolates previously isolated from various strains of *Nocardia* (Maurice *et al.*, 1971; Alshamaony *et al.*, 1976; Yano *et al.*, 1978). Table 2 shows the molecular weights, the nature of branched chains and the unsaturation of these esters. The corresponding acids contained 52 to 56 carbon atoms with two double bonds.

The components of spot C (R_F 0.34) had the same migration as corynomycolic methyl esters isolated from corynebacteria. The fragment peaks obtained on MS of these esters are indicated in Table 3.

The peaks at m/z 518 to 464 correspond to anhydromycolates ($M - 18$). Two series of peaks in the m/z range 505 to 451 and m/z 486 to 432 correspond to $M - 31$ and $M - 50$ ions, respectively. Peaks at m/z 270, 242 and m/z 299, 271 result from the fragmentations a and b and indicate the presence of a side-chain ($C_{12}H_{25}$ or $C_{14}H_{29}$; Maurice *et al.*, 1971). These results were confirmed by chemical ionization MS in the presence of isobutane (Fig. 2). The peaks at m/z 511, 509 and 483 correspond to ions $M + H$. The major fragment ions are found at m/z 493, 491 and 465 ($M - 18 + H$) and at m/z 479, 477 and 451 ($M - 32 + H$).

Pyrolytic GC of methyl corynomycolates (Etémadi, 1967) gave methyl esters of C_{14} and C_{16} fatty acids and C_{16} , $C_{18:0}$ and $C_{18:1}$ aldehydes. Methyl corynomycolates were quantified by gas chromatographic analysis of the trimethylsilyl ether derivatives. The percentages of corynomycolic acids were different in both strains: 15% in R1316 and 37% in R416 of the total fatty acids.

Thus the strains R416 and R1316 of *N. otitidis-caviarum* have two types of mycolic acids in their neutral lipids: nocardomycolic acids (C_{52} to C_{56}) and corynomycolic acids (C_{30} to C_{34}). Corynomycolic acids consist of saturated and monounsaturated compounds with C_{12} and C_{14} side-chains; the major component was the C_{32} acid (11% in R1316, 25% in R416).

Fig. 1. TLC of fatty acid methyl esters from strains of *N. otitidis-caviarum*. Adsorbent, silica gel G; solvent system, hexane/diethyl ether (4:1, v/v); detection, bromothymol blue/sodium hydroxide solution. Lanes: 1, methyl corynomycolates; 2, 4 and 5, fatty acid methyl esters of strain R1316, IM 1380 and R416, respectively; 3 and 6, fatty acid methyl esters from the cell wall of strains R1316 and R416, respectively; 7, standard nocardomycolates. A, Non-hydroxylated fatty acid methyl esters; B, nocardomycolates; C, corynomycolates; X, unidentified compound.

Table 2. Peaks in the mass spectra of methyl nocardomycolates

Strain	Fragment ions (<i>m/z</i>)				Side-chain (R')	Formula of acids	No. of double bonds
	<i>a</i> ₁	<i>a</i> ₂	<i>b</i>	M-18			
<i>N. otitidis-caviarum</i> R1316	242	516	271	768	C ₁₂ H ₂₅	C ₅₂ H ₁₀₀ O ₃	2
	270	544	299	796	C ₁₄ H ₂₉	C ₅₄ H ₁₀₄ O ₃ *	2
		572		824		C ₅₆ H ₁₀₈ O ₃	2
<i>N. otitidis-caviarum</i> R416	242	516	271	768	C ₁₂ H ₂₅	C ₅₂ H ₁₀₀ O ₃	2
	270	544	299	796	C ₁₄ H ₂₉	C ₅₄ H ₁₀₄ O ₃ *	2
	298	572	327		C ₁₆ H ₃₃		

* Major compound.

Table 3. Peaks in the mass spectra of methyl corynomycolates

Strain	Fragment ions (<i>m/z</i>)					Side-chain	Formula of acids	No. of double bonds
	<i>a</i> ₁ *	<i>b</i> *	M-50	M-31	M-18			
<i>N. otitidis-caviarum</i> R1316	242	271	432	451	464	C ₁₂ H ₂₅	C ₃₀ H ₆₀ O ₃	0
	270	299	458	477	490	C ₁₄ H ₂₉	C ₃₂ H ₆₂ O ₃	1
			460	479	492		C ₃₂ H ₆₄ O ₃ †	0
<i>N. otitidis-caviarum</i> R416	242	271	432	451	464	C ₁₂ H ₂₅	C ₃₀ H ₆₀ O ₃	0
	270	299	458	477	490	C ₁₄ H ₂₉	C ₃₂ H ₆₂ O ₃	1
			460	479	492		C ₃₂ H ₆₄ O ₃ †	0
			486	505	518		C ₃₄ H ₆₆ O ₃	1

* For the fragment ions *a*₁ and *b* see Table 2.

† Major compound.

Fig. 2. Isobutane chemical ionization mass spectrum of methyl corynomycolates from *N. otitidis-caviarum* R1316.

Localization of mycolic acids in cells

The fatty acids prepared from the cell walls of strains R416 and R1316 were esterified by diazomethane and analysed by TLC (Fig. 1). In both strains, only nocardomycolic acids were found as components of cell walls, corynomycolic acids being absent.

Distribution of mycolic acid types

The nature of mycolic acids in various strains of *N. otitidis-caviarum* was determined by TLC of methyl esters of fatty acids from total lipids. The R_F values of corynomycolic and nocardomycolic methyl esters in three solvents were respectively 0.34 and 0.44 in hexane/diethyl ether (4:1, v/v), 0.32 and 0.40 in hexane/acetone (19:1, v/v), and 0.34 and 0.40 in hexane/diethyl ether/acetic acid (160:40:1, by vol.).

Mycolic acid patterns of 16 strains of *N. otitidis-caviarum* were examined: 11 strains (IM 1381, IM 1380, N939, N940, N942, N943, N36, N314, N5, N6) contained only nocardomycolic acids, four strains (R1316, R416, IM 1379, IMRU 1355) had both types of mycolic acids and one strain (IMRU 1259) contained only very small amounts of corynomycolic acids.

DISCUSSION

The presence of both types of mycolic acid in *N. otitidis-caviarum* is not exceptional, as five among sixteen strains studied contained more or less important amounts of corynomycolic acids. Itoneda *et al.* (1970) had already pointed out the presence of trehalose corynomycolates in a strain of *N. asteroides*. In our study the strains which had both types of mycolic acid had only nocardomycolic acids in the cell walls. Probably there is a selection in the pool of mycolic acids for the biosynthesis of polysaccharide mycolates which constitute a part of the cell wall polymers. For the strains containing both types of mycolic acids there is a clear discontinuity in the sizes of these acids with the nocardo-type having about 52 to 56 carbon atoms and the coryno-type having about 30 to 34 carbon atoms; no intermediates were found. If nocardomycolic acids are synthesized by elongation of the carbon chain followed by a mycolic condensation (Etémadi & Lederer, 1965), this process must be regulated by a very precise mechanism. The mycolic condensation is effective only when a well defined size of the hydrocarbon chain is reached: C_{16} to C_{18} for corynomycolic acids, C_{30} to C_{40} for nocardomycolic acids. Certain strains of *Nocardia*, therefore, possess the enzymic equipment for both types of mycolic acid condensation reactions.

All the representatives of *N. otitidis-caviarum* and other species of *Nocardia* already studied contained nocardomycolic acids, regardless of the presence or absence of corynomycolic acids. Thus, nocardomycolic acids remain good chemotaxonomic markers for the genus *Nocardia*. Chemical ionization is a useful alternative to electron impact ionization for the mass spectrometric identification of mycolic acid methyl esters.

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