Journal of Chromatography, 487 (1989) 142–146
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4496

Note

Gas chromatographic fatty acid determination to differentiate *Nocardia asteroides*, *Mycobacterium fortuitum* and *Mycobacterium chelonei*

J. FOURCHE*

Laboratoire de Bactériologie, Service Professeur Latrille, Hôpital Xavier-Arnozan, 33600 Pessac (France)

M. CAPDEPUY

Laboratoire de Microbiologie Pharmaceutique, Service Professeur Guinet, Université de Bordeaux II, Jardin Botanique, 33400 Talence (France)

and

J. TEXIER-MAUGEIN

Laboratoire de Bactériologie, Service Professeur Latrille, Hôpital Xavier-Arnozan, 33600 Pessac (France)

(First received June 2nd, 1988; revised manuscript received September 13th, 1988)

The atypical mycobacteria are classified into four groups; usually most of these bacteria are consisted as harmless saprophytes. Group IV is composed of the rapid growers among which two species should now be considered as emerging pathogens, *Mycobacterium fortuitum* and *M. chelonei*, just like the bacteria of the *Nocardia* genus.

The use of corticosteroids and other immunosuppressive therapy is associated with an increase of unusual infections due to these bacteria; they are recognized with increasing frequency in patients with AIDS [1,2].

The *Mycobacterium* and *Nocardia* genera belong to the Actinomycetales family; their cell-wall lipids and long-chain mycolic acids are closely related. Morphological differentiation between *Nocardia* and rapid-growing mycobacteria species is sometimes difficult because the cells of these mycobacteria may be less than 10% acid-fast and those of nocardiae are also only slightly acid-fast. Their identification is based on morphological, cultural and biochemical criteria [3]. The distinguishing feature of *Nocardia* genus is the rudimentary to extensively branched vegetative hyphae. However, growth medium composition and culture

0378-4347/89/$03.50

conditions can affect this morphological feature. Differential identification of *Nocardia* species is based upon cultural and biochemical characteristics, but these do not give highly reproducible results. *M. chelonei* differs from *M. fortuitum* in its inability to reduce nitrate and to utilize fructose, and in a few other less used tests [4–6].

During recent years, a number of studies have shown that it is possible to distinguish between bacteria species on the basis of their long-chain cellular fatty acids. After extraction and methylation, the cellular fatty acids can be identified by gas chromatography (GC) [7–9]. This present investigation examines the long-chain fatty acids (C_{12} and longer) and cleavage products of mycolic acids of *Nocardia* and *Mycobacterium* species by GC and mass spectrometry (MS). The specific markers are identified.

EXPERIMENTAL

Microorganisms

The cultures examined in this study included the following strains:

M. fortuitum: one reference strain, ATCC 6843; two strains obtained from the Laboratory of the Pitié Salpêtrière Hospital (Paris, France) (courtesy of Professor J. Grosset); six strains isolated from clinical specimens at the Centre Hospitalier Régional (CHR) (Bordeaux, France).

M. chelonei: four reference strains, *M. chelonei* subsp. *chelonei*, ATCC 19236, NCTC 946 and *M. chelonei* subsp. *abcessus*, ATCC 19977, ATCC 14472; two strains from Professor J. Grosset; five strains from the CHR (Bordeaux, France).

N. asteroides: one reference strain, IP 320, from Pasteur Institute (Paris, France); one strain from Pasteur Institute (Lyon, France); seven strains from clinical specimens (Bordeaux, France).

N. caviae: one reference strain, IP 776, from Pasteur Institute (Paris, France).

N. brasiliensis: one reference strain, IP 337, from Pasteur Institute (Paris, France).

The strains isolated from biological samples were identified according to standard criteria.

Extraction

Bacteria were cultivated on Löwenstein–Jensen media until growth was sufficient for analysis (generally five days).

Approximately 100 mg of bacteria were collected by a platinum wire and heated at 105 °C for 90 min in 5 ml of a 7.5 *M* solution of sodium hydroxide in methanol–water (6:4, v/v). After cooling, 6 ml of distilled water were added and the mixture was acidified (pH 2) by the addition of a 20% (v/v) sulphuric acid aqueous solution. The liberated fatty acids were then extracted once with 6 ml of diethyl ether. The ether extracts were washed with distilled water to obtain a neutral aqueous phase and dried over anhydrous sodium sulphate. The ether extracts were then evaporated. Methyl esters were formed by adding 2 ml of a boron trifluoride–methanol solution (20%, v/v) for 10 min at 100 °C; they were extracted

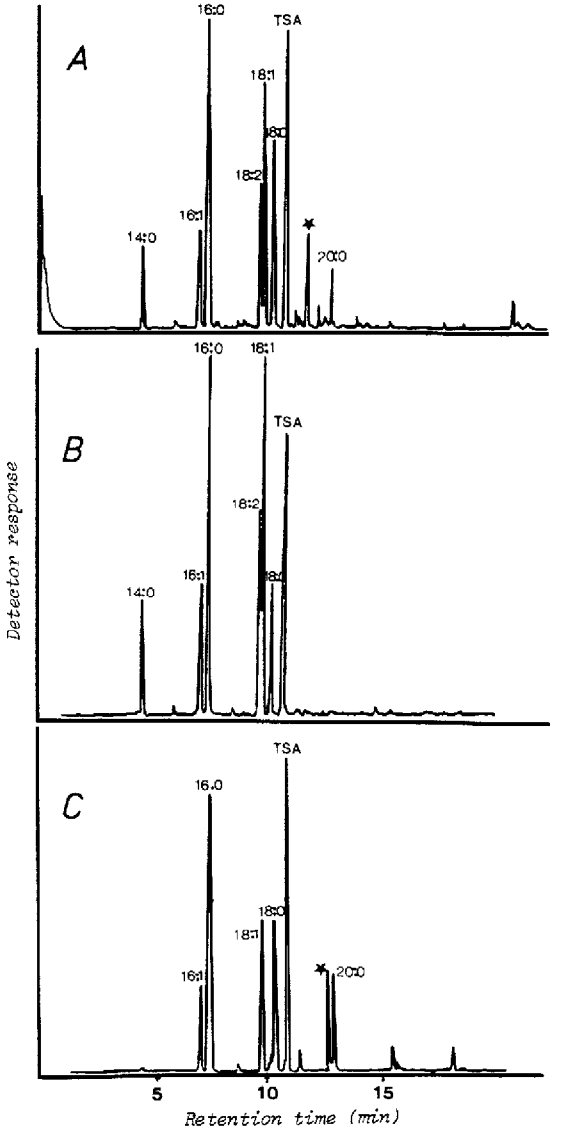

Fig. 1. Gas chromatograms of the fatty acid methyl esters of *Mycobacterium fortuitum* ATCC 6843 (A), *Mycobacterium chelonei* subsp *abcessus* ATCC 14472 (B) and *Nocardia asteroides* IP 320 (C). ⋆ = specific markers.

using 2 ml of chloroform–*n*-hexane (1:4, v/v). The organic phase was evaporated under nitrogen and the extracts were stored at −4 °C.

Gas chromatography–mass spectrometry

The extracts were analysed on a Packard 427 gas chromatograph equipped with a flame ionization detector. Analyses were performed on a SPB1 fused-silica capillary column (15 m × 0.53 mm I.D., $d_f = 1.5$ μm; Supelco, Bellefonte, PA, U.S.A.). The temperatures of the injector and the detector were 280 and 320 °C, respec-

tively. The oven temperature was programmed from 140 to 230°C at a rate of 4°C/min. The extracts were dissolved in 0.1 ml of *n*-hexane, and 2 µl were injected.

The fatty acid methyl esters were identified by comparing their retention times with those of standard products: these standards were a bacterial acid methyl ester mixture (4-5436; Supelco). Identification of peaks not corresponding to standards was made by MS on a Spectro-magnetic VG-Micromass 16 F (LTD, Winsford, U.K.).

RESULTS

Fig. 1 shows the chromatograms for *M. fortuitum* (A), *M. chelonei* (B) and *N. asteroides* (C). All the strains contained the most common fatty acids (palmitic, palmitoleic, stearic, oleic, linoleic) and tuberculostearic acid (TSA or 10-methyl-octadecanoic acid). The presence of other peaks seems very important because there are more specific.

(a) The *M. fortuitum* species contained a compound that eluted after TSA and before eicosanoic acid. This compound was found in all the tested strains (reference and clinical specimen strains) but neither in *M. chelonei* nor in *Nocardia*.

(b) The *N. asteroides* strains gave a peak just before eicosanoic acid; this was not observed in *N. caviae* or in *N. brasiliensis*, and was absent in *M. chelonei* and *M. fortuitum*. This compound was found in every chromatogram of *N. asteroides*, but in various amounts according to the strain examined.

These two specific peaks were identified by MS, and the corresponding spectra are shown in Fig. 2. According to the different major fragment ions observed and to the literature [10], the fatty acid detected in *M. fortuitum* was tentatively identified to 2-methyl-2-octadecenoic acid (as methyl ester, Fig. 2A). The specific compound in *N. asteroides* is an eicosenoic acid methyl ester (Fig. 2B).

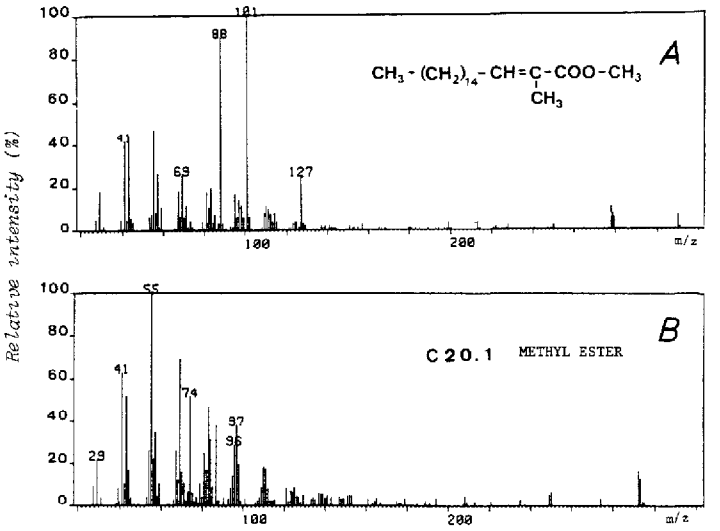

Fig. 2. Mass spectra of the specific fatty acids (as methyl esters) of *Mycobacterium fortuitum* (A) and *Nocardia asteroides* (B).

DISCUSSION

Differentiation between *M. fortuitum* and *M. chelonei* using biochemical tests is not easy, so that several authors studied the cellular fatty acid composition. Tisdall and co-workers [11,12] were unable to differentiate *M. fortuitum* from *M. chelonei* species, but Daffé et al. [13] pointed out that the mycolic acids of these two species are different. Larsson et al. [14] and Lambert et al. [15] noted but did not identify a specific peak eluted before eicosanoic acid in *M. fortuitum* chromatograms. Recently, Valero-Guillén et al. [16] have reported and identified this compound in *M. fortuitum*, but neither in *M. chelonei* nor in *M. phlei*. We agree with them that this 2-methyl-2-octadecenoic acid may serve as a chemical marker for *M. fortuitum* identification.

Data on *Nocardia* cellular fatty acids are rare: Tisdall et al. [11] and Lechevallier et al. [17] have observed that members of *Nocardia* genus do not contain C_{24} and C_{26} fatty acids.

The specific eicosenoic acid we have identified in all the *N. asteroides* strains may be a marker that differentiates this pathogenic *Nocardia* from other species and from the rapid-growing non-chromogenic mycobacteria. All these mycobacteria we have studied contain a myristic acid peak, which has not been found in *Nocardia* species; this makes it possible to differentiate *M. chelonei* from *N. caviae* and *N. brasiliensis*, bacteria that have no specific markers.

This study suggests that lipid analysis is a very suitable method for identifying the most commonly encountered *Nocardia* strains and two species of rapid-growing mycobacteria, *M. fortuitum* and *M. chelonei*. It would be interesting to use this method for the diagnosis of other atypical mycobacteria and for the detection of these bacterial markers directly in body fluids and specimens.

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