Mass spectrometry as a tool for identifying group D2 corynebacteria by their fatty acid profiles

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Corynebacterium group D2 (CGD2) are lipophilic antibiotic-multiresistant bacteria involved in some infections of immunocompromised patients. The fatty acid composition and structure of different strains was established by several mass spectrometric methods, particularly negative ion tandem mass spectrometry coupled with capillary gas chromatography. Non-hydroxylated fatty acid profiles of three strains of CGD2 (ATCC 43042, ATCC 43043, ATCC 43044) were almost identical and revealed the presence of several straight chain unsaturated fatty acids from the ω -9 series, with even carbon numbers ranging from 14 to 24. Branched saturated fatty acids were mainly anteiso-heptadecanoic acid and tuberculostearic acid. Surprisingly, a relatively large quantity of 10-methylene octadecanoic acid was found. The non-hydroxylated fatty acid profile of one rare β -lactam susceptible strain (SC1) was different; 10-methylene octadecanoic acid was lacking whereas tuberculostearic acid was much more abundant. In contrast, the four CGD2 strains displayed highly similar mycolic acid patterns. The major mycolic acid species corresponded to C32, C30 and C28 bis-unsaturated with a double bond on each branch at the ω -9 position. The comparison of the mycolic acid composition and structure with those of other medically important corynebacteria strains, revealed a characteristic pattern for CGD2 strains, and CGD2 strains were easily distinguished from *Corynebacterium jeikeium* (CIP 82.51).

Introduction

The analysis of total cellular fatty acid composition has proved useful for the classification and identification of bacteria (Minnikin & Goodfellow, 1980; Daffé *et al.*, 1983; Athalye *et al.*, 1984), particularly for coryneform and related taxa (Jones & Collins, 1986). The structures of mycolic acids, together with the nature of branched and straight chain fatty acids, have been used for taxonomic studies (Corina & Sesardic, 1980; Minnikin & Goodfellow, 1980; Collins *et al.*, 1982; Suzuki & Komagata, 1983). The position of double bonds in unsaturated fatty acids also has taxonomic significance (Suzuki *et al.*, 1982).

Corynebacterium group D2 (CGD2) (King, 1972) are lipophilic antibiotic-multiresistant bacteria. They are potential pathogens in immunocompromised patients and have been involved in urinary tract infections (Soriano *et al.*, 1988; Soriano & Fernandez-Roblas, 1988; De Briel *et al.*, 1991) and in one case of endocarditis (Langs *et al.*, 1988). Although the GC profile of the fatty acids from CGD2 has been presented already (Soriano *et al.*, 1988), together with their mycolic acid composition

(Herrera-Alcaraz *et al.*, 1990), several components remained undetermined. The work described in this paper used a new mass spectrometry approach (Savagnac *et al.*, 1989), allowing the precise characterization of several unknown components. Furthermore, the mycolic acid profile was shown to differ from that already described. However, a comparison with other coryneform bacteria grown in the same conditions, showed a characteristic mycolic acid pattern for CGD2.

Methods

Strain cultures. The following strains were obtained from the American Type Culture Collection (ATCC), Rockville, MD, USA, the National Collection of Type Cultures (NCTC), London, UK, and the Collection of the Institut Pasteur (CIP), Paris, France: *Corynebacterium* group D2 ATCC 43042, ATCC 43043, ATCC 43044, *C. pilosum* ATCC 29592 (type strain), *C. xerosis* ATCC 373 (type strain), *C. bovis* NCTC 3324 (type strain), *C. minutissimum* NCTC 10288 (type strain), *C. diphtheriae* CIP 78.02, *C. jeikeium* (C. group JK) CIP 82.51, *C. mycetoides* CIP 55.51 (type strain), *C. pseudotuberculosis* CIP 52.97, *C. renale* CIP 69.37. The rare β -lactam-susceptible strain of *Corynebacterium* D2 (strain SC1) was isolated as previously reported (De Briel *et al.*, 1991).

All bacteria were grown on 5% (v/v) sheep blood agar (trypticase soy agar, BioMérieux, France) supplemented with Tween 80 (1%, v/v; Merck, FRG) and incubated at 37 °C for 72 h. This medium was derived from a selective-enrichment medium (De Briel *et al.*, 1991). Cultures were checked for purity.

Isolation and separation of fatty acids. Cells (300 mg wet wt) were harvested using a wire loop, and were hydrolysed using 2 ml 25% (w/v) KOH/50% (v/v) ethanol overnight at 85 °C. The mixture was then extracted with 2 ml chloroform after acidification with 1.5 ml 6 M-HCl. The chloroform layer was evaporated to dryness and hydrolysed again using 2 ml 5% (w/v) KOH in 50% (v/v) methanol to remove ethyl esters formed during the first hydrolysis. The free acids were methylated with diazomethane by addition of a few drops of an ethereal solution of diazomethane until the yellow colour remained. Excess reagent was destroyed with a dilute solution of acetic acid in diethyl ether.

Non-hydroxylated fatty acid esters and mycolic acid esters were separated on a silicic acid HPLC column (nucleosil-50, 5 µm, 100 × 4–6 mm, SFCC, France) using a 95:5 cyclohexane/ethyl acetate solvent mixture. Trimethylsilyl (TMS) ether derivatives were synthesized by dissolving approximately 0.1 mg methyl mycolate in 0.5 ml bis-silyltrifluoroacetamide (BSTFA) containing 0.1% trimethylchlorosilane (TMCS). The reaction was complete after 3 h at room temperature. Pentafluorobenzyl (PFB) derivatives were synthesized by mixing approximately 1 mg free fatty acids with pentafluorobenzyl-bromide (2 µl), diisopropylethylamine (2 µl), acetonitrile (50 ml) and methanol (10 µl). The mixture was stirred at room temperature for 1 h (Promé *et al.*, 1987).

Mass spectrometry. Mass spectrometry was performed on a reverse geometry BE mass spectrometer (ZAB-HS, VG Analytical, Manchester, UK) fitted with a combined electron impact/chemical ionization (EI/CI) ion source. The accelerating voltage was 8 kV.

Gas liquid chromatography coupled with electron impact mass spectrometry. The mass spectrometer was interfaced with a DANI 3800 gas chromatograph. An aluminium-sheathed fused silica column (0.33 mm i.d., 12 m length, coated with 0.1 µm HT5 phase, SGE, Ringwood, Victoria, Australia) was used for separations. The gas chromatographic system was connected to the ion source through a polyimide-sheathed deactivated fused silica column (0.05 mm i.d., 20 cm length) to ensure electrical insulation. This interface was heated at 270 °C, and helium was the carrier gas. The mass scan speed was 0.8 s per decade, and the injection was carried out using a Ross injector.

Non-hydroxylated fatty acid methyl esters were analysed directly (oven temperature rising from 80 to 250 °C at 2 °C min⁻¹). The injector temperature was 250 °C.

Mycolic acid esters were first converted into their O-TMS ether derivatives as described above, and were then injected at 270 °C (oven temperature rising from 140 to 350 °C at 2 °C min⁻¹).

Electron-capture negative ion tandem mass spectrometry. The PFB esters of the non-hydroxylated fatty acids were characterized by combined capillary GC-tandem mass spectrometry (GC/MS/MS) working in the negative ion mode. The CI source was filled with methane, and a 70 eV electron beam was used. The mass spectrum of each PFB ester was very simple, since it only exhibited the carboxylate ion. A preliminary GC/MS experiment was carried out to determine both the molecular mass of each carboxylate anion and the retention time of the corresponding PFB.

In a second experiment, the collision cell located between the magnet and the electrostatic analyser was filled with helium. The magnet was set to transmit the carboxylate anion of each species, which appeared at

the expected retention time. The electric voltage was scanned repetitively at a speed of 1.5 s per cycle, and all spectra were collected and stored. The kinetic energy spectra were converted into mass spectra by computer calculation (Courcelle *et al.*, 1989).

PFB esters of corynomycolic acids were analysed in a similar way, except that the direct inlet probe was used. Each carboxylate anion was successively focused on the gas cell, by adjusting the magnet to the appropriate field while scanning the electrostatic voltage.

Results

The negative ion GC/MS/MS method

Negative ion electron-capture ionization of fatty acid PFB esters dissociated them into the corresponding carboxylate anions and the PFB radical (Reaction 1).

Reaction 1

Upon collision activation, the carboxylate anions were cleaved at each carbon-carbon bond by a charge-remote fragmentation process, the charge remaining on the carboxylate moiety (Tomer *et al.*, 1983; Adams, 1990). Since vinylic and double bonds were fragmented more slowly than other bonds, a double bond could be located by three consecutive weak peaks surrounded by two intense ones, due to faster cleavage of allylic bonds. A methyl branch was located by the absence of a single peak in the sequence of regularly spaced ones. Fatty acids containing both double bonds and alkyl branches were easily characterized, which is relatively difficult using other mass spectrometric methods. This method was greatly improved by direct coupling with capillary GLC, which allowed the molecular separation of almost all the fatty acid isomers (Promé *et al.*, 1987, 1990; Couderc *et al.*, 1989a).

Profile and structure of the non-hydroxylated fatty acids of CGD2

Palmitic acid is the major straight chain saturated fatty acid of CGD2 (Fig. 1). Small amounts of tetradecanoic, pentadecanoic, heptadecanoic, stearic and tetraeicosanoic acids were also detected. Branched saturated fatty acids were identified as tuberculostearic acid (Fig. 2b) and *anteiso*-heptadecanoic acid (Fig. 2a), together with small amounts of *iso*-fatty acids.

Straight chain unsaturated fatty acids of the ω-9 series were identified, namely Δ5-tetradecenoic, Δ7-hexadecenoic, Δ9-octadecenoic, Δ11-eicosenoic and Δ15-

Fig. 1. GC of PFB ester derivatives of the non-hydroxylated fatty acids from *Corynebacterium* D2, strain ATCC 43043. For abbreviations of the fatty acid nomenclature, see legend to Table 1. Identification: (a) C14:1 Δ 5; (b) C14:0; (c) C15:0 i; (d) C15:0 a; (e) C15:0 n; (f) C16:1 Δ 7; (g) C16:1 Δ 9; (h) C16:1 Δ 11; (i) C16:0 n; (j) C17:0 i; (k) C17:0 a; (l) C17:1 Δ ^R₈; (m) C17:0 n; (n) C18:1 Δ 9; (o) C18:0 n; (p) C19:0 R₁₀; (q) C19:1 Δ ^R₁₀; (r) C20:1 Δ 11; (s) C24:1 Δ 15; (t) C24:0 n; (x) non-fatty acids.

tetracosenoic acids (Fig. 2d). Surprisingly, a relatively large quantity of 10-methylene octadecanoic acid was found (Fig. 2c). This peculiar structure was clearly deduced from the pattern between the two abundant ions at m/z 195 and m/z 141 (allylic cleavages) where the middle weak signal was lacking (Couderc *et al.*, 1989b). This methylene-branched fatty acid is an intermediate in tuberculostearic acid biosynthesis (Lenfant *et al.*, 1966; Akamatsu & Law, 1970). Only minute amounts have been detected in *Mycobacterium* (Promé *et al.*, 1990) and it has not been previously reported in lipids of *Corynebacterium*.

Three strains of CGD2 (ATCC 43042, ATCC 43043, ATCC 43044) exhibited similar fatty acid profiles (Table 1). The only difference was quantitative; the abundance of both tuberculostearic acid and 10-methylene octadecanoic acid was lower in strain ATCC 43042 than in strains ATCC 43043 and ATCC 43044. The SC1 strain gave a different profile: 10-methylene octadecanoic acid was not detected and tuberculostearic acid was found to be two to ten times more abundant than in the other strains. Thus, although three strains of CGD2 exhibited a characteristic non-hydroxylated fatty acid profile, the fourth strain had a similar profile to several other corynebacteria and could not be differentiated in this way.

Table 1. Fatty acid composition of four different strains of CGD2

i, iso type; a, anteiso type; n, linear type; R_j, methyl branched; and Δ ^R_j, methylene branched on carbon number j. Percentages correspond to the relative area of the peaks using a flame ionization detector

Fatty acid	CGD2 ATCC 43042 (%)	CGD2 ATCC 43043 (%)	CGD2 ATCC 43044 (%)	CGD2 SC1 (%)
C14:1 Δ 5	7.1	6.0	7.5	2.9
C14:0	2.3	1.1	1.0	1.5
C15:0 i	1.5	0.4	6.3	0.4
C15:0 a	0.5	0.6	0.4	0.8
C15:0 n	0.4	0.4	0.3	1.1
C16:1 Δ 7	8.5	7.4	6.5	3.5
C16:1 Δ 9	2.8	2.3	1.6	2.1
C16:1 Δ 11	2.2	2.1	2.2	2.9
C16:0 i	0.1	0.5	0.3	0.4
C16:0 n	16.3	16.1	15.8	16.9
C17:0 i	0.5	0.5	0.7	1.4
C17:0 a	2.2	2.8	3.1	5.7
C17:0 n	0.6	0.3	0.8	1.3
C17:1 Δ ^R ₈	0.1	0.6	0.1	—
C18:1 Δ 9	39.1	34.2	29.9	40.3
C18:0 n	2.5	1.4	1.4	1.3
C19:0 R ₁₀	1.6	7.4	8.1	16.3
C19:1 Δ ^R ₁₀	3.5	7.5	5.6	—
C20:1 Δ 11	0.7	0.4	0.5	1.2
C24:1 Δ 15	6.3	6.6	6.1	—
C24:0 n	1.2	1.4	1.8	—

Fig. 2. CAD-MIKE spectra of carboxylate anions from several fatty acids. The separation of the corresponding PFB derivatives was shown in Fig. 1. (a) C17:0 a (peak k); (b) C19:0 R₁₀ (peak p); (c) C19:1 ΔR₁₀ (peak q); (d) C16:1 Δ7 (peak f). For fatty acid nomenclature, see Table 1.

Fig. 3. Corynomycolic acid composition of (a) SC1, (b) ATCC 43042, (c) ATCC 43043, (d) ATCC 43044. ■, Saturated; ▨, monounsaturated; □, diunsaturated.

Composition and structure of mycolic acids of CGD2 and comparisons with other strains

The EI/GC/MS analysis of the TMS ether derivatives of the methyl mycolates of the four strains of CGD2 was carried out as described by Yano *et al.* (1972) and Chevalier *et al.* (1988). Each species was characterized by the presence of the (M-15)⁺ ion which allowed the

Fig. 4. CAD-MIKE spectrum of the carboxylate anion at m/z 491 from the C32 diunsaturated corynomycolic acid (CGD2, strain ATCC 43043).

determination of both total carbon content and the number of double bonds. Fragmentation arising from cleavage of the C2–C3 bond gave the chain length of the alkyl-branch and its degree of unsaturation. Major species corresponded to C32, C30 and C28 diunsaturated mycolic acids. Monounsaturated mycolic acids were less abundant and were observed mainly among the C28 analogues (Fig. 3).

The 2-alkyl branch consisted mainly of a dodecenyl group. The 3-hydroxylated main chain contained either zero or one double bond. Its length ranged from C16 (C28 mycolic acids) to C20 (C32 mycolic acids).

The position of the double bonds was assigned by negative ion MS/MS (i.e. the charge-remote fragmentation process). Because of the low volatility of PFB ester derivatives of mycolic acids, on-line GC separation was not attempted. Thus, the MS/MS analyses were carried out by direct introduction of these derivatives.

The mycolic acid carboxylate anion MS/MS spectra were dominated by abundant cleavages of the C2–C3 bonds. However, regularly spaced weaker signals due to remote-charge fragmentations were also observed. Examination of the latter allowed the localization of the double bonds. Since both chains were cleaved in parallel, each spectrum was the superimposition of the fragmentation patterns from each chain (Savagnac *et al.*, 1989). The observed spectra from the diunsaturated species were consistent with the exact superimposition of the patterns from the two double bonds, without any shift relative to each other (Fig. 4). This demonstrated that the

double bonds were located at the same distance from each methyl end, at the ω -9 position. The same location was observed in the monounsaturated species.

The mycolic acid composition of some medically important corynebacteria is presented in Fig. 5. The comparison of the structure of CGD2 with these corynebacteria (Figs 3 and 5, respectively) revealed both a homogeneous and a specific overall GC pattern for the D2 group.

Discussion

The fatty acid composition and profile from CGD2, cultivated under conditions different from our own, has been recently described (Soriano *et al.*, 1988; Herrera-Alcaraz *et al.*, 1990). However, several components were identified only by comparison with authentic standards. Using capillary GC, coupled with negative ion ionization and MS/MS, we accurately identified all the non-hydroxylated fatty acids. It has been reported (Soriano *et al.*, 1988) that CGD2 contains both tuberculostearic and tuberculopalmitic acids. We confirmed the presence of the former, but found only traces of the latter. Furthermore, the presence of 10-methylene octadecanoic acid, quantitatively equivalent to tuberculostearic acid, was unexpected in the genus *Corynebacterium*, since it has previously been detected only in trace amounts, in the genus *Mycobacterium*. We could only detect trace amounts of tuberculopalmitic acid. However, a relatively

Fig. 5. Comparison of the corynomycolic acid patterns from several coryneform bacteria. ■, Saturated; ▨, monounsaturated; □, diunsaturated.

large amount of *anteiso*-heptanoic acid was present among the C17 branched fatty acids. Moreover, we also observed fatty acids with longer carbon chains, e.g. Δ^{15} -tetracosenoic acid, which is an unusual feature in corynebacteria. Most of the straight chain unsaturated fatty acids detected belong to the ω -9 series. The exception was the C16:1 species in which small amounts of ω -5 and ω -7 unsaturated fatty acids were also present. The presence of many unsaturated fatty acids from the ω -9 series may be consistent with their biosynthesis via an anaerobic-like pathway involving the elongation of a *cis* Δ^3 -dodecenoic acid, as proposed for several coryneform bacteria (Suzuki *et al.*, 1982). However, it has been reported that several *Corynebacterium* strains were able both to hydrolyse Tween 80, used to supplement the culture medium, and to use its oleic acid part for the synthesis of fatty acids (Chevalier *et al.*, 1988). Thus, the origin of ω -9 unsaturated fatty acids from the CGD2 stains may be due to elongation or shortening of oleic acid from Tween 80.

Structures of mycolic acids from coryneform bacteria have received a great deal of attention and have been used as a tool for the identification of medically important strains (Yano *et al.*, 1972; Minnikin *et al.*, 1978; Athalye *et al.*, 1984, 1985). It has been shown that corynebacteria can take up fatty acids from the culture medium, and that its composition can influence the nature of the mycolic acids synthesized (Chevalier *et al.*, 1988). Thus, all comparisons between different strains of corynebacteria must be under standardized cultivation conditions. Hence, we examined several coryneform strains grown on the same medium supplemented with Tween 80. The mycolic acids from the four CGD2 strains exhibited very similar compositions, but their pattern was different from all other coryneform strains. Particularly, a distinction from *Corynebacterium jeikeium* could be achieved by examination of the mycolic acid composition. It was notable that the mycolic acid composition of CGD2 we observed was clearly different from that recently described by Herrera-Alcaraz *et al.*

(1990), particularly regarding the number of double bonds. This is probably due to different culture conditions. Nevertheless, provided identical growth conditions are used for all strains, the mycolic acid pattern seems a convenient tool for the identification of coryneforms including *Corynebacterium* D2.

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