Gas Chromatography–Mass Spectrometry of Mycolic Acids as a Tool in the Identification of Medically Important Coryneform Bacteria

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The mycolic acid derivatives of 11 unidentified pathogenic coryneform bacteria were examined by TLC, GLC and GLC–mass spectrometry. The resulting mycolic acid profiles of the unidentified isolates were compared with those of type or reference strains of possibly related coryneform species, namely *Corynebacterium bovis*, *C. diphtheriae*, *C. xerosis* and *Rhodococcus equi*. It was apparent that most of the unidentified strains showed a distinctive mycolic acid profile, with predominant amounts of relatively high molecular weight mycolic acids (C₃₂–C₃₆) and a high degree of unsaturation, and could thus be distinguished from both *C. bovis*, which had exceptionally low molecular weight mycolic acids (C₂₄–C₃₀), and *C. diphtheriae* (C₂₈–C₃₄), which had large amounts of saturated mycolic acids. The mycolates of *C. xerosis* and *R. equi* (C₂₈–C₃₆) were generally similar to those of the unidentified coryneforms but their overall mycolic acid patterns were different from one another as well as the unidentified strains. The mycolic acid profiles exhibited by the pathogenic coryneforms examined here were very similar to one another but unlike that of any of the type or reference strains included in the study.

INTRODUCTION

Lipid analyses are now well established in bacterial taxonomy and have provided good characters for the classification and identification of many bacterial genera (Goldfine, 1972; Lechevalier, 1977; Lechevalier *et al.*, 1971; Minnikin & Goodfellow, 1976, 1980; Minnikin *et al.*, 1978, 1980; Shaw, 1974). Mycolic acids, which are 2-alkyl,3-hydroxy long, branched fatty acids, have been found to be of particular value in the classification of actinomycetes with a wall-chemotype IV (Becker *et al.*, 1965), that is with *meso*-diaminopimelic acid (*meso*-DAP), arabinose and galactose (Collins *et al.*, 1982; Corina & Sesardic, 1980; Goodfellow & Minnikin, 1977; Goodfellow *et al.*, 1982; Minnikin *et al.*, 1978, 1980). The mycolic acids of mycobacteria are complex in composition and may contain components with oxygen functions in addition to the 3-hydroxy system while the mycolic acids from other sources such as corynebacteria, nocardiae and rhodococci are simpler by comparison, comprising homologous mixtures of saturated and unsaturated components containing between 20 and 60 carbon atoms (Goodfellow & Minnikin, 1977; Lechevalier, 1977; Minnikin & Goodfellow, 1976, 1980; Minnikin *et al.*, 1978, 1980; Yano *et al.*, 1972, 1978).

We have obtained a number of pathogenic coryneform bacteria (Table 1) that have been shown to be relatively biochemically inactive and thus have proved difficult to identify using conventional diagnostic tests (Applebaum & Dossett, 1981; Gronemeyer *et al.*, 1980; Hande *et*

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al., 1976; Hill *et al.*, 1978; Hine *et al.*, 1978; Johnson & Kaye, 1970; Kaplan & Weinstein, 1969; Riley *et al.*, 1979; Stamm *et al.*, 1979). These organisms, variously referred to as 'Corynebacterium group JK' (Applebaum & Dossett, 1981; Gill *et al.*, 1981; Gronemeyer *et al.*, 1980; Hande *et al.*, 1976; Riley *et al.*, 1979; Stamm *et al.*, 1979) and 'Corynebacterium bovis-like group' (Hill *et al.*, 1978; Hine *et al.*, 1978), have often been found to cause serious complications in subjects who either had a debilitating systemic disease or were receiving immunosuppressive drug therapy following major surgery. Prominent among this group were subjects with clinical evidence of endocarditis (Murray *et al.*, 1980; Van Scoy *et al.*, 1977) or who had undergone bone marrow transplants (Stamm *et al.*, 1979). Also included in this group were patients with lymphadenitis, osteomyelitis, wound infections, meningitis, pneumonia and empyema (Applebaum & Dossett, 1981; Gill *et al.*, 1981; Gronemeyer *et al.*, 1980; Hande *et al.*, 1976; Johnson & Kaye, 1970; Kaplan & Weinstein, 1969; Riley *et al.*, 1979). Prognosis in such cases is often very poor. Furthermore, recent evidence suggests that many of these pathogenic isolates are resistant to a wide range of antibiotics (Gill *et al.*, 1981; Gronemeyer *et al.*, 1980; Riley *et al.*, 1979), thus accurate identification of these organisms may greatly improve the treatment and prognosis of infections attributed to them.

This paper describes a GLC and GLC-mass spectrometry (GLC-MS) analysis of *tert*-butyldimethylsilyl (*t*-BDMS) derivatized mycolic acids from 11 pathogenic coryneform bacteria. The mycolic acid profiles of the unidentified strains were compared with the patterns from reference strains of possibly related species of coryneform bacteria such as *Corynebacterium bovis*, *C. diphtheriae*, *C. xerosis* and *Rhodococcus equi* (Collins *et al.*, 1982; Corina & Sesardic 1980; Goodfellow *et al.*, 1982, 1983; Hill *et al.*, 1978; Hine *et al.*, 1978).

METHODS

Strains and cultivation. Cultures (Table 1), obtained from private and public culture collections, were routinely maintained on nutrient agar (Oxoid) supplemented with 0.5% (v/v) horse serum (Oxoid) and 0.2% (v/v) Tween-80 (BDH). The test strains were cultivated on the same medium, and after incubation at 30 °C for 2–3 d, the cells were harvested, killed, washed and then dried as described elsewhere (Corina & Sesardic, 1980). Cells of the reference culture *C. diphtheriae* PW8 (NCTC 4305) were supplied by A. F. B. Standfast, Lister Institute of Preventative Medicine, Elstree, UK.

Table 1. *Pathogenic coryneform and reference strains examined for their detailed mycolic acid profiles*

Code	Identification	Source‡
C6, C79*†	<i>C. bovis</i>	NCDO 1931 and NCTC 3224
PW8†	<i>C. diphtheriae</i>	NCTC 4305
C96*†	<i>C. xerosis</i>	ATCC 373
C2, C78*†	<i>R. equi</i>	NCDO 1927 and NCTC 1621
C63, C65, C67	' <i>Corynebacterium</i> JK'	003, 005 and 119; isolated from bone marrow transplant patients, Seattle, USA
C103, C105	' <i>Corynebacterium</i> sp.'	554 and 5110; isolated from bone marrow transplant patients, London, UK
C10	'atypical <i>C. bovis</i> '	NCTC 17/66; synovial membrane of right atrium
C25	'atypical <i>C. bovis</i> '	NCTC A111/74; blood of SBE patient
C28	'atypical <i>C. bovis</i> '	NCTC A136/74; blood of renal transplant patient
C133	' <i>Corynebacterium</i> sp.'	E123; blood of SBE patient
C136	' <i>Corynebacterium</i> sp.'	A81; blood of renal transplant patient
C137	' <i>Corynebacterium</i> sp.'	G4989; blood of patient after transurethral resection of carcinoma of the prostate

* Indicates the type strain of the species.

† Indicates strains whose mycolic acids have been examined in detail elsewhere (Collins *et al.*, 1982; Corina & Sesardic, 1980; Gailley *et al.*, 1982; Minnikin *et al.*, 1978).

‡ ATCC, American Type Culture Collection, Rockville, Md., USA; NCDO, National Collection of Dairy Organisms, Shinfield, Reading, UK; NCTC, National Collection of Type Cultures, Central Public Health Laboratory, Colindale, London, UK; SBE, Sub-acute endocarditis.

Extraction and derivatization of mycolic acids. Dried cells (50 mg) were degraded by acid methanolysis (Minnikin *et al.*, 1980) and the presence of mycolic acid methyl esters was detected by analytical TLC (Minnikin *et al.*, 1980). Methyl mycolates were purified by preparative TLC and converted to the corresponding *t*-BDMS ethers (Corey & Venkateswarlu, 1972) by dissolving in dry toluene (0.15 ml) and heating in a closed tube at 55 °C for 4 h with a reagent (0.15 ml) prepared from *t*-butyldimethylsilyl chloride (0.15 g) and imidazole (0.17 g) in dimethyl formamide (1 ml). The *t*-BDMS ethers were extracted from the cooled reaction mixture with petroleum ether, b.p. 60–80 °C (3 × 0.3 ml) and the pooled extracts purified by passing through a small (0.5 cm) diethyl ether-washed column of neutral alumina (BDH). The purified extracts were evaporated to dryness under a stream of nitrogen at 37–42 °C and stored at 0 °C.

Analysis of *t*-BDMS ethers of mycolates by GLC. The purified *t*-BDMS ethers of mycolates were analysed using a Packard-430 gas chromatograph equipped with a fused silica capillary column, 0.24 mm × 25 m, coated with OV-101 (Chrompack UK; code no. 'CP-Sil 5'). The *t*-BDMS ethers of mycolates were dissolved in 8–10 drops of petroleum ether and 2 µl of the solution was injected into the chromatograph. The split injection ratio was set at 50:1. In all cases, an identical temperature program was employed in which the initial temperature of 280 °C was held for 3 min before being raised to 320 °C at a rate of 3 °C min⁻¹. The separated peaks were tentatively identified on the basis of their retention times as compared to those from *C. diphtheriae* PW8 and were later confirmed by GLC–MS analysis.

Confirmation of peak assignments. GLC peak assignments were verified by GLC–MS analysis. Two different columns were used: a 2 mm × 1 m glass column packed with 1% OV-101 on Chromosorb W-HP, and a 0.3 mm × 12 m quartz capillary column coated with SE-30 (Hewlett Packard; 'methyl silicone' wall-coated column). Both types of columns were operated at the appropriate temperatures. The mass spectrometer was a VG Analytical 305 magnetic sector instrument with an associated 2025 data system. Electron impact ionization was used and in all cases the ionization potential was 40 eV. The identity of the majority of the separated peaks was confirmed by selected ion recording (SIR) for which the accelerating voltage switching mode was used.

RESULTS

TLC analysis of the acid methanolysates showed the presence of mycolic acid methyl esters in all of the strains examined (Table 1). The methyl mycolates exhibited a relatively low R_f value (R_f 0.2–0.4) which indicated that they contained between 20 and 40 carbon atoms (Minnikin & Goodfellow, 1976; Minnikin *et al.*, 1980).

The *t*-BDMS derivatives of methyl mycolates were prepared to facilitate the analysis of their structures. Figure 1 shows the GLC traces obtained for the mixtures of *t*-BDMS ethers of methyl mycolates extracted from '*Corynebacterium* group JK' C67 and *C. bovis* C6, respectively. As can be seen, the different molecular species of mycolic acids were all well separated, and their provisional identity and relative concentrations of the original mixture could be determined with relative ease.

Peaks were initially identified on the basis of their relative retention times and on earlier reports (Collins *et al.*, 1982; Corina & Sesardic, 1980; Gailley *et al.*, 1982; Minnikin *et al.*, 1978). The identity of the peaks was confirmed later by GLC–MS analysis. A partial mass spectrum of the C_{32:0} methyl mycolate-*t*-BDMS ether from *C. diphtheriae* PW8 is shown in Fig. 2. Little fragmentation takes place in these compounds and the spectra are characterized by a base peak corresponding to the [M-57]⁺ ion and by two ions, (a) and (b) in Fig. 2, which are produced by the two possible fragmentations α to the *t*-BDMS group.

The eluants from capillary gas chromatography columns were monitored at the m/z values expected for the [M-57]⁺ ions for the various mycolic acid derivatives. By this method a complete identification, including the degree of unsaturation, of the different components was made, and in all cases these confirmed the tentative identifications made on the GLC analysis alone. The mycolic acids from some of the reference strains included in the present study have been investigated before (Collins *et al.*, 1982; Corina & Sesardic, 1980; Gailley *et al.*, 1982; Minnikin *et al.*, 1978; 1980) and our results are in full agreement with these reports.

The results are presented in Table 2 where strains are grouped according to the approximate relationships indicated by their complete mycolic acid profiles. Thus, the type strain of *C. bovis* (C79), the NCDO reference strain of *C. bovis* (C6) and two unidentified pathogenic coryneform strains, C103 and C136, are placed together because all four strains had mycolic acids with 24–30 carbon atoms. Their overall mycolic acid profiles were also very similar.

Fig. 1. GLC analysis of *t*-BDMS ethers of methyl mycolates of (a) *Corynebacterium* group JK' C67 and (b) *C. bovis* C6 on CP-Sil 5. On each peak, the first number indicates the number of carbon atoms and the second indicates the number of double bonds.

Fig. 2. Partial mass spectrum of the mycolic acid, identified as $C_{32:0}$, from *C. diphtheriae* PW8. (a) and (b) are referred to in the text.

Table 2. Confirmed mycolic acid contents (% of total) of coryneform bacteria examined

Carbon chain length	Base peak [M-57] ⁺ m/z	Number of double bonds	<i>C. bovis</i>	<i>C. bovis</i>	<i>C. bovis</i>	<i>Corynebacterium</i> sp.	<i>C. diphtheriae</i>	<i>C. xerosis</i>	<i>Corynebacterium</i> sp.	<i>R. equi</i>	<i>R. equi</i>	<i>Corynebacterium-JK*</i>	<i>Corynebacterium-JK*</i>	<i>Corynebacterium-JK*</i>	'atypical <i>C. bovis</i> '	<i>C28</i>	'atypical <i>C. bovis</i> '	<i>C105</i>	<i>Corynebacterium</i> sp.	<i>C137</i>
<i>C</i> ₂₄	449	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	451	2	1.9	t	2.8	-	-	-	-	2.4	2.9	-	-	-	-	-	-	10.4	-	4.6
	453	1	10.5	9.6	8.0	t	t	t	4.2	12.8	1.7	-	-	-	-	-	-	-	-	-
	455	0	1.2	1.9	2.0	1.8	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>C</i> ₂₆	477	3	t	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	479	2	9.1	7.9	3.5	4.5	t	-	-	2.5	-	-	-	-	-	-	-	-	-	-
	481	1	13.8	11.4	8.9	10.8	t	t	3.1	4.0	2.1	-	-	-	t	-	-	-	-	-
	483	0	5.2	2.6	3.1	2.9	4.8	-	t	-	-	-	-	-	2.5	2.5	-	1.5	-	t
<i>C</i> ₂₈	505	3	1.3	t	4.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	507	2	5.6	t	2.2	6.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	509	1	33.5	32.9	34.1	32.7	t	-	t	11.3	2.7	-	-	-	1.6	2.7	-	-	-	-
	511	0	-	-	-	4.6	-	3.4	2.2	4.8	-	-	3.8	5.4	1.4	2.3	-	-	-	-
<i>C</i> ₃₀	533	3	t	3.7	t	3.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	535	2	8.7	6.0	10.9	8.6	-	3.7	5.9	-	-	-	-	-	1.4	3.1	1.6	4.3	1.7	-
	537	1	3.2	7.4	6.6	5.4	2.9	t	3.6	-	-	1.7	1.7	2.6	3.4	3.3	2.0	1.5	1.5	-
	539	0	-	-	-	20.6	-	7.2	1.7	-	-	-	3.2	3.0	1.0	3.8	-	4.0	4.2	-
<i>C</i> ₃₂	561	3	5.4	8.4	8.8	5.3	-	-	-	-	3.0	41.0	48.4	33.6	3.8	34.2	35.6	44.1	47.7	-
	563	2	-	2.3	6.4	1.3	3.6	27.1	30.3	-	2.2	3.7	4.0	3.2	-	3.0	t	7.5	3.3	-
	565	1	-	-	-	-	7.6	6.8	11.1	6.8	10.4	t	1.0	t	3.2	t	-	4.3	2.8	-
	567	0	-	-	-	38.7	-	-	-	2.1	-	-	-	-	t	-	-	-	-	-
<i>C</i> ₃₄	589	3	-	-	-	-	-	-	-	12.4	30.5	10.9	5.7	7.3	45.4	2.2	12.9	7.5	2.1	-
	591	2	-	-	-	-	-	4.2	13.8	-	17.6	6.1	5.4	3.5	t	6.2	10.5	5.3	9.5	-
	593	1	-	-	-	-	1.7	10.4	10.8	4.7	6.7	2.1	2.3	t	21.1	4.6	1.4	t	t	-
	595	0	-	-	-	8.8	-	6.1	t	-	-	-	-	-	-	-	-	-	-	-
<i>C</i> ₃₆	617	3	-	-	-	-	-	3.8	t	1.0	t	-	-	-	-	-	-	-	-	-
	619	2	-	-	-	-	-	17.3	13.4	29.1	13.0	19.0	12.2	34.7	11.5	16.3	29.4	14.7	20.0	-
	621	1	-	-	-	-	-	8.9	t	4.2	3.7	2.0	3.0	t	2.1	3.8	t	t	t	-
	623	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

* Indicates the type strain of the species; t indicates trace or not integrated; italicized entries indicate the predominant mycolic acid in each strain.

DISCUSSION

Mycolic acids from corynebacteria and related organisms have been examined in detail using a variety of methods (Collins *et al.*, 1982; Corina & Sesardic, 1980; Gailley *et al.*, 1982; Minnikin *et al.*, 1978; Yano *et al.*, 1972, 1978). Complete mycolic acid patterns of named corynebacteria, nocardiae and rhodococci have now been obtained by the analysis of methyl mycolates by mass spectrometry (Collins *et al.*, 1982; Minnikin *et al.*, 1978) and of trimethylsilyl derivatives of methyl mycolates by both GLC and GLC-MS (Corina & Sesardic, 1980; Gailley *et al.*, 1982; Tomiyasu, 1982; Yano *et al.*, 1972, 1978). The use of *t*-BDMS ethers of methyl mycolates is also an attractive technique since such derivatives are stable to moisture and give relatively simple mass spectra with intense ions suitable for rapid and sensitive SIR.

Closer examination of Table 2 shows that of the 11 unidentified pathogenic coryneforms included in the study, two, C103 and C136, were grouped with *C. bovis* and one, C133, with *C. diphtheriae* and *C. xerosis*. However, the remaining eight strains previously identified as 'Corynebacterium group-JK' (Hande *et al.*, 1976; Stamm *et al.*, 1979) or '*C. bovis*-like' (Hill *et al.*, 1978; Hine *et al.*, 1978), fell into a closely related group which also included the type strain of *R. equi*, C78, and an NCDO reference strain, C2, previously identified as *R. equi* (Hill *et al.*, 1978). Thus, our results indicate that the majority of pathogenic coryneform bacteria provisionally identified as '*C. bovis*-like' (Hill *et al.*, 1978) in fact showed little similarity to the type or reference strains of *C. bovis*, but did have mycolic acid profiles that were practically identical to the 'Corynebacterium group-JK' strains. The results also show that this distinct group of strains could be distinguished from all the type and reference strains of *Corynebacterium* included in the study. Thus, our results support the conclusions of Hande *et al.* (1976) and Stamm *et al.* (1979) which suggest that these organisms may well constitute a new species.

However, the data presented here also show that this group of pathogenic coryneform organisms has a mycolic acid profile somewhat similar to the type and reference strains of *R. equi*. This indicates that further studies are necessary to establish the relationship of the unidentified strains to corynebacteria and rhodococci. Numerical phenetic studies of rhodococci have already shown that although *R. equi* is a typical member of the genus *Rhodococcus*, its mycolic acid profiles overlap those of certain corynebacteria and some rhodococci (Goodfellow *et al.*, 1982, 1983). A comprehensive numerical taxonomic study of a large number of unidentified pathogenic coryneform bacteria, together with strains from possibly related *Corynebacterium* and *Rhodococcus* species, involving biochemical, morphological and chemical characters would provide further valuable data for the classification of the as yet unidentified pathogenic organisms. The analysis of mycolic acid profiles by the procedure described here does, however, provide a sensitive and relatively easy method for the preliminary characterization of these important pathogens.

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