A Survey of the Structures of Mycolic Acids in *Corynebacterium* and Related Taxa

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Acid methanolysates of 104 strains of coryneform and related bacteria were examined for mycolic acids by thin-layer chromatography and mass spectrometry. The mycolic acids of *Corynebacterium sensu stricto* strains were not uniform in size and structure, and considerable variation existed within the approximate limiting range of C₂₂ to C₃₆. Two species, *Corynebacterium bovis* and '*Corynebacterium mycetoides*', were particularly distinctive; the former had exceptionally low molecular weight mycolic acids (C₂₂ to C₃₂), whereas the latter contained major amounts of mycolates with a side-chain possessing an odd number of carbon atoms. *Caseobacter polymorphus* contained mycolic acids of a similar size (C₃₀ to C₃₆) to those of true corynebacteria. The mycolates of organisms assigned to the genus *Rhodococcus* were generally larger (C₃₀ to C₅₆), although a clear distinction between true corynebacteria and rhodococci cannot presently be made by analysis of mycolic acids alone. Strains labelled '*Arthrobacter roseoparaffinus*', *Corynebacterium equi* and *Corynebacterium hoagii* contained mycolic esters with molecular weights 'intermediate' in size between those of true corynebacteria and rhodococci. The mycolic acid data correlate well with other major trends in coryneform taxonomy and support earlier suggestions that they are of value in the classification of the group.

INTRODUCTION

Despite improvements in the classification of coryneform and related bacteria due to numerical phenetic analyses (Jones, 1978), DNA base composition determinations (Crombach, 1972; Starr *et al.*, 1975) and cell wall analyses (Schleifer & Kandler, 1972; Keddie & Cure, 1977, 1978; Keddie & Bousfield, 1980), many problems remain. Attention has increasingly turned to lipid analyses for the provision of characters for the classification of coryneform and related taxa (Goodfellow *et al.*, 1976; Yamada *et al.*, 1976; Collins & Jones, 1980; Collins *et al.*, 1977, 1979a, b, 1980; Keddie & Cure, 1977; Minnikin *et al.*, 1978, 1979).

Thin-layer chromatographic analysis of mycolic acids, i.e. long-chain 2-branched 3-hydroxy fatty acids, has been particularly useful in clarifying the taxonomy of the genera *Corynebacterium*, *Mycobacterium*, *Nocardia* and *Rhodococcus* (Goodfellow *et al.*, 1976; Keddie & Cure, 1977; Minnikin *et al.*, 1975; Minnikin & Goodfellow, 1980). Structural studies indicate that mycobacteria usually contain a complex mixture of mycolic acids of high

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molecular weight (C_{60} to C_{90}) whereas those from nocardiae and rhodococci are smaller (C_{36} to C_{66}) (Alshamaony *et al.*, 1976*a, b*; Minnikin & Goodfellow, 1980). Although the structure of mycolic acids in true corynebacteria has not been the subject of systematic analyses, preliminary studies performed to date suggest that some corynebacteria possess mycolic acids of a relatively low molecular weight (C_{20} to C_{36}) (see Minnikin *et al.*, 1978). Furthermore, mycolic acids of similar size have been isolated from strains labelled '*Arthrobacter paraffineus*' (Suzuki *et al.*, 1969), '*Brevibacterium thiogenitalis*' (Okazaki *et al.*, 1969), '*Bacterionema matruchotii*' (Alshamaony *et al.*, 1977) and '*Mycobacterium laticolum* var. *aliphaticum*' (Krasilnikov *et al.*, 1973).

In the present study the general structures of the mycolic acids of a large number of coryneform bacteria have been investigated.

METHODS

Cultures and cultivation. Test strains (Table 1) were maintained routinely on nutrient agar (Oxoid) and Dorset egg slopes at room temperature. Strains were grown in shake culture at 30 °C for 3 d in nutrient broth no. 2 (Oxoid). Cultures were checked for purity, killed by shaking with formalin, harvested by centrifugation (10 000 g for 10 min), washed with distilled water and freeze-dried.

Whole-organism methanolysis and thin-layer chromatography. Dried organisms (50–100 mg) were degraded by acid methanolysis as described by Minnikin *et al.* (1975), and the hexane extracts were examined for long-chain components by thin-layer chromatography using Merck silica gel H (0.5 mm layers) and a developing mixture of petroleum ether (b.p. 60–80 °C)/diethyl ether (85:15, v/v). Mycolic acid methyl esters were isolated by preparative thin-layer chromatography on layers (1 mm) of Merck silica gel PF₂₅₄₊₃₆₆ using the same developing mixture, detected by illumination with long wave ultraviolet light (366 nm) and eluted from the gel with chloroform.

Pyrolysis gas chromatography. Pyrolysis gas chromatography of mycolic acid methyl esters was carried out on a Perkin-Elmer F11 flame-ionization gas chromatograph fitted with stainless steel columns (0.125 in, o.d.). Analyses were performed on 2 m non-polar OV-1 (2.5% OV-1 silicone on 80–100 mesh Chromosorb G AW-DMCS; Phase Separations) and 6 m Silar 10C (10% Silar 10C on 100–120 mesh Gas-Chrom Q; Applied Science Laboratories) columns using nitrogen as carrier gas at isothermal oven temperatures of 180 °C and 210 °C, respectively. Components in the chromatograms were identified by comparing their retention times with those of authentic long-chain esters and aldehydes.

Mass spectrometry. Mass spectra of mycolic esters were taken on an AEI MS9 instrument using a direct insertion probe, an ionizing voltage of 70 eV and a temperature of 160–200 °C.

Table 1. *Mycolic acid-containing test strains and their sources*

Name as received†	No. of strains	Strain designation(s)/source‡
' <i>Arthrobacter albidus</i> '	1	D6 (NCIB 10266)
' <i>A. hydrocarboglutamicus</i> '	1	C795 (ATCC 15583)
' <i>A. paraffineus</i> '	1	C228 (NCIB 10699)
' <i>A. picolinophilus</i> '	1	C905 (ATCC 27854)
' <i>A. roseoparaffinus</i> '	1	C112 (NCIB 10700)
' <i>A. variabilis</i> '	1	*D2 (NCIB 9455)
' <i>Brevibacterium ammoniagenes</i> '	1	C80 (NCIB 8143)
' <i>B. butanicum</i> '	1	C790 (ATCC 21196)
' <i>B. chang-fua</i> '	1	C794 (ATCC 14017)
' <i>B. divaricatum</i> '	1	D3 (NCIB 9379)
' <i>B. flavum</i> '	1	C81 (NCIB 9565)
' <i>B. glutamigenes</i> '	1	C900 (ATCC 13747)
' <i>B. heali</i> '	1	C903 (ATCC 15527)
' <i>B. immariophilum</i> '	1	*C123 (NCIB 9544)
' <i>B. ketoglutamicum</i> '	1	C260 (DSM 20165)
' <i>B. lactofermentum</i> '	1	C124 (NCIB 9567)
' <i>B. paraffinolyticum</i> '	1	C113 (NCIB 11160)
' <i>B. roseum</i> '	1	C82 (NCIB 9564)
' <i>B. saccharolyticum</i> '	1	C127 (NCIB 9543)
' <i>B. stationis</i> '	1	*C41 (ATCC 14403)

Table 1. (cont.)

Name as received†	No. of strains	Strain designation(s)/source‡
' <i>B. sterolicum</i> '	1	C188 (NCIB 11161)
' <i>B. taipei</i> '	1	C796 (ATCC 13744)
<i>Caseobacter polymorphus</i>	4	*C357 (NCDO 2097); C358 (NCDO 2099); C359 (NCDO 2101); C360 (NCDO 2102)
' <i>Corynebacterium acetoacidophilum</i> '	1	C129 (NCIB 9661)
' <i>C. alkanum</i> '	1	C901 (ATCC 21194)
<i>C. bovis</i>	9	*C12 (NCTC 3224); C95 (NIRD 1689); C97 (NIRD 1928); C98 to C103 (J. Schreeve: DB223/75, DB132/75, DB210/75, 30/75, DB94/75, 120B)
<i>C. callunae</i>	1	*C130 (NCIB 10338)
<i>C. diphtheriae</i>	5	C13 (NCTC 3985); C14 (NCTC 3987); C32 (NCTC 3984); C66 (NCTC 3529); C67 (NCTC 10681)
<i>C. equi</i>	1	*C7 (NCTC 1621)
<i>C. fascians</i>	3	*C1 (ATCC 12974); C104 (NCPPB 1488); N146 (M. Turner: 39)
' <i>C. flavidum</i> '	1	C35 (NCTC 764)
<i>C. glutamicum</i>	1	D7 (NCIB 10025)
' <i>C. herculis</i> '	1	*C85 (NCIB 9694)
<i>C. hoagii</i>	1	C24 (NCTC 10673)
' <i>C. hydrocarboclastus</i> '	2	*D8, D9 (K. Komagata: AJ 1386, AJ 1379)
<i>C. kutscheri</i>	3	C20 (NCTC 949); C59 (NCTC 1386); C60 (NCTC 3655)
<i>C. lilium</i>	1	C133 (NCIB 10337)
' <i>C. melassecola</i> '	1	C136 (NCIB 10336)
' <i>C. minutissimum</i> '	2	*D24 (NCTC 10288); D24B (NCTC 10285)
' <i>C. mycetoides</i> '	1	C63 (NCTC 9864)
<i>C. pseudodiphtheriticum</i>	2	C19 (NCTC 231); C37 (D. Jones: C10)
<i>C. pseudotuberculosis</i>	14	C15 (NCTC 3450); C214 (NCTC 4655); C215 (NCTC 4681); C216 (NCTC 4691); C142, C150 to C156 (H. R. Carne: Ov 133, Ov 1134, Ov 1139, Cap 16, Cap.16-F302-4; Cap.16-F302-35, E107, E1144); H5, H7 (P. Maximescu: 992, 993)
<i>C. renale</i>	4	*C17 (NCTC 7448); H1, H2, H4 (R. Yanagawa, 43, 45, 42)
' <i>C. rubrum</i> '	1	*C190 (NCIB 9433)
' <i>C. segmentosum</i> '	1	C64 (NCTC 934)
' <i>C. ulcerans</i> '	10	C18 (NCTC 7910); C170, C172, C174, C176, C178, C179, C180 (H. R. Carne: JW2, JW7, JW33, JW40, PM39C, PM298G, PM319C); H6, H8 (P. Maximescu: 896, 985)
<i>C. xerosis</i>	3	C27 (NCTC 7238); C137 (NCIB 9956); C33 (NCTC 9755)
<i>Corynebacterium</i> sp.	4	D11, D16, D17, D18 (I. J. Bousfield: marine isolates SN66, SN123, SN149, SN130)
' <i>Microbacterium ammoniaphilum</i> '	1	C139 (NCIB 10335)
' <i>Microbacterium flavum</i> '	1	D4 (NCIB 8707)
' <i>Mycobacterium lacticolum</i> '	1	C237 (NCIB 9740)
' <i>Mycobacterium rubrum</i> subsp. <i>propanicum</i> '	1	C239 (NCIB 9741)

* Type strain.

† Taxa which are not included in the *Approved Lists of Bacterial Names* (Skerman *et al.*, 1980) are shown in quotation marks.

‡ ATCC, American Type Culture Collection, Rockville, Maryland, U.S.A.; DSM, Deutsche Sammlung von Microorganismen, Teilsammlung, 61 Darmstadt, West Germany; NCIB, National Collection of Industrial Bacteria, Aberdeen, U.K.; NCPPB, National Collection of Plant Pathogenic Bacteria, Harpenden, Herts, U.K.; NCTC, National Collection of Type Cultures, London, U.K.; NCDO, National Collection of Dairy Organisms, Shinfield, Reading, U.K.; NIRD, National Institute for Research in Dairying, Shinfield, Reading, U.K.; I. J. Bousfield, Torry Research Station, Aberdeen, U.K.; H. R. Carne, University of Cambridge, U.K.; D. Jones, Department of Microbiology, University of Leicester, U.K.; K. Komagata, Ajinomoto Co., Kawasaki, Japan; P. Maximescu, Dr I. Cantacuzino Institute, Bucharest, Rumania; J. Shreeve, Bacteriology Department, Central Veterinary Laboratory, New Haw, Weybridge, U.K.; M. Turner, University of Nottingham, U.K.; R. Yanagawa, Hokkaido University, Sapporo, Japan. C, D, H and N are laboratory numbers.

Fig. 1. Pyrolysis gas chromatographic analysis of mycolic acid methyl esters of *Corynebacterium xerosis* C33 on OV-1 (profile *a*) and Silar 10C (profile *b*). On each peak, the first number indicates the number of carbon atoms, the second indicates the number of double bonds and the final letter indicates whether it is an aldehyde (a) or ester (e).

RESULTS AND DISCUSSION

Thin-layer chromatographic analysis of the whole-organism methanolysates revealed the presence of mycolic acid methyl esters (R_F 0.2 to 0.5) in all of the test strains (Table 1). Pyrolysis gas chromatography of the mycolic acid methyl esters from *Corynebacterium xerosis* C33 was performed in order to clarify the interpretation of the mass spectra of the mycolic esters under consideration. The analyses were performed on both polar and non-polar stationary phases: the results are shown in Fig. 1. Pyrolysis gas chromatography of mycolic esters yields mixtures of long-chain esters and aldehydes (Etémadi, 1967; Lechevalier *et al.*, 1973), and from the mycolates of *C. xerosis* C33 complex mixtures of mainly monounsaturated components were observed (Fig. 1).

Methyl esters of mycolic acids fragment on mass spectrometry in several competing pathways depending on their overall size and structural features (Etémadi, 1967; Maurice *et al.*, 1971; Alshamaony *et al.*, 1976*a, b*; Minnikin & Goodfellow, 1976, 1980; Goodfellow *et al.*, 1978) as summarized in Fig. 2. The highest peaks in the mass spectra of methyl mycolates correspond to anhydromycolates formed by the elimination of the elements of water from the parent molecule. Cleavage according to *a* and *a'* (Fig. 2) involves net transfer of the hydroxyl hydrogen atom to give long-chain aldehydes (meroaldehydes) and esters. Unsaturated esters produced by cleavage *a* do not give prominent peaks but readily lose the elements of methanol to give characteristic fragments X (Fig. 2; Goodfellow *et al.*, 1978). Fragments Y due to cleavage *b* complement those arising from *a* and assist in determining the overall size of the side-chain R_1 (Fig. 2).

In the present study, peaks at m/e 158, 186, 214, 242, 256, 270, 284 and 298 correspond to saturated esters (Fig. 2; cleavage *a*) and fragments at m/e 187, 215, 243, 271, 285, 299, 313 and 327 (Fig. 2; cleavage *b*) confirm the presence of side-chains R_1 (Fig. 2) having the overall elemental composition C_6H_{13} , C_8H_{17} , $C_{10}H_{21}$, $C_{12}H_{25}$, $C_{13}H_{27}$, $C_{14}H_{29}$, $C_{15}H_{31}$ and

Fig. 2. Mass spectral fragmentation pathways for mycolic acid methyl esters.

 Fig. 3. Partial mass spectrum of mycolic acid methyl esters of *Corynebacterium xerosis* C33.

$\text{C}_{16}\text{H}_{33}$, respectively. The presence of side-chains $\text{C}_{14}\text{H}_{27}$ and $\text{C}_{16}\text{H}_{31}$, containing one double bond, was shown by the presence of small peaks at m/e 268/297 and 296/325 and larger fragments at m/e 236 and 264 formed by elimination of the elements of methanol from the parent ester (Fig. 2). The intensities of the last peaks (X^+ , Fig. 2), however, are variable due to the presence of other competing reactions and do not accurately reflect the proportions of monounsaturated side-chains (R_1) in the parent mycolates.

The variety of competing pathways (Fig. 2) and the similarity of the size of the fragments occurring in the mass spectra of the mycolic esters under study make it difficult to propose accurate structures based on mass spectral analysis alone. In the partial mass spectrum of the mycolic acid methyl esters of *C. xerosis* C33 (Fig. 3), for example, prominent peaks at m/e 516 and 544 are due to the main components of anhydromycolates (Fig. 2) corresponding to diunsaturated C_{34} and C_{36} parent mycolic acids, respectively. Smaller peaks can be assigned to the minor series of anhydromycolates (Fig. 3) and other peaks, m/e 484 and 512 for example, are due to loss of the elements of methanol from the major anhydromycolates.

The complex array of peaks in the region m/e 200–350 of the mass spectrum of the mycolates from *C. xerosis* C33 (Fig. 3) is mainly due to fragments including the side-chain R_1 (Fig. 2). Peaks at m/e 236 and 264 (Fig. 3) do correspond in size, as noted previously (Alshamaony *et al.*, 1977), to diunsaturated aldehydes, but on pyrolysis gas chromatography no such aldehydes were detected. Monounsaturated esters lose the elements of methanol

[illegible]

Table 2. (cont.)

Structure of R ₁ branch*	C ₈ H ₁₇	C ₁₀ H ₂₁	C ₁₂ H ₂₅	C ₁₃ H ₂₇	C ₁₄ H ₂₇		C ₁₄ H ₂₉	C ₁₅ H ₃₁	C ₁₆ H ₃₁		C ₁₆ H ₃₃
'C. segmentosum' C64		+			+	tr	+		+++	+	
'C. ulcerans' C18		+		+	+	tr	+++		++	+	
'C. ulcerans' C170		+		+	+	+	+++	+			
'C. ulcerans' C172		+		+	+	+	+++	+	+	+	
'C. ulcerans' C174		+		+	+	+	+++	+	tr	tr	tr
'C. ulcerans' C176		+		+	+	+	+++	tr	tr	tr	tr
'C. ulcerans' C178		+		+	+	+	+++	+	tr		
'C. ulcerans' C179		++		+	+	+	+++	+	tr		
'C. ulcerans' C180		++		+	+	+	+++		++		
'C. ulcerans' H6		+++		tr	+	tr	+++		++		
'C. ulcerans' H8		++		tr	tr	tr	+++	tr	++	+	
C. xerosis C27		+		tr	tr	+	+++		+++		
C. xerosis C33		++			++	+	+++		+		+
C. xerosis C137		tr		+	+	tr	+++	tr	+	tr	
Corynebacterium sp. D11		+			+	tr	+++		+		
Corynebacterium sp. D16		+			+	tr	+++		+		
Corynebacterium sp. D17		+			+	tr	+++	tr	++	+	+
Corynebacterium sp. D18					+	tr	+++		++	+	+
'Microbacterium ammoniaphilum' C139					+	+	+++	+	+	+	+
'Microbacterium flavum' D4				+	+	tr	+++		+		
'Mycobacterium laticolium' C237		+	++	+	tr	tr	+++		+		
'Mycobacterium rubrum subsp. propanicum' C239		+	++	+	+	tr	+++		+		

* Explanation of assignment of peaks is given in Results.

† Peaks at *m/e* 268 and 296 are attributable to C_{16:1} and C_{18:1} esters, respectively; peaks at *m/e* 236 and 264 are due to the loss of the elements of methanol (32 mass units) from these esters.

Table 3. Peaks in the mass spectra of methyl mycolates from *Corynebacterium bovis* strains corresponding to anhydromycolates

The main component of each series is denoted by +++, any component greater than 50% of the main peak by ++, and all other significant components by +.

No. of carbons in parent mycolic acid	<i>m/e</i>	No. of double bonds	<i>C. bovis</i>								
			C12	C95	C97	C98	C99	C100	C101	C102	C103
C ₂₂	350	1					+	+			
C ₂₃	364	1			+		++	+			
C ₂₄	376	2	+					++			
	378	1	+	++	++	+	+++	+++	+	+	+
C ₂₅	390	2	+	+	+	+	+	+	+	+	+
	392	1	+	+	+	+	++	++	+	+	+
C ₂₆	404	2			+		+	+			
	406	1	+	++	++	++	++	++	+	++	++
C ₂₇	418	2	+	++	+	+	+	+	+	++	+
	420	1	++	++	++	++	+	++	++	++	++
C ₂₈	432	2	+	++	++	++	+	+	+	++	+
	434	1	+++	+++	+++	+++	++	++	+++	+++	+++
C ₂₉	446	2	+	+	+	+	+	+	+	+	+
	448	1	+	+	+	+	+	+	+	+	+
	450	0		+				+			
C ₃₀	460	2	+	+	+	+	+	+	+	+	+
	462	1	+		+	+	+	+	+	+	+
C ₃₁	474	2	+	+	+	+			+	+	+
	476	1	+	+	+	+			+	+	+
C ₃₂	488	2	+	+	+	+				+	+
	490	1	+		+	+				+	+

much more readily than saturated esters (Campbell & Naworal, 1969; Goodfellow *et al.*, 1978) and the peaks at *m/e* 236 and 264 must be such products formed from monounsaturated C₁₆ and C₁₈ esters. Peaks in the mass spectra attributable to aldehydes were not clearly identifiable for the mycolates under study. The detection of aldehyde fragments was also unreliable in the spectra of mycolates from nocardiae and rhodococci (Alshamaony *et al.*, 1976*a, b*), but for mycobacterial mycolates the peaks due to aldehydes were consistently recorded (Etémadi, 1967). The series of peaks corresponding to anhydromycolates were, however, very reliable in appearance. Interpretation of the mass spectra of the mycolic acid methyl esters from the test organisms, bearing in mind the complexities demonstrated for *C. xerosis* C33, enabled us to determine the distribution of the side-chains R₁ (Table 2) and the anhydromycolate compositions (Tables 3 to 6).

The mycolic acids from *Corynebacterium bovis* strains were particularly distinctive (Table 3) having a carbon range of C₂₂ to C₃₂ and side-chains R₁ of the composition C₆H₁₃ and C₈H₁₇ (major component). The presence of such small parent acids and side-chains R₁ distinguishes *C. bovis* from all other corynebacteria examined.

Corynebacterium diphtheriae strains contained mycolic acids with carbon ranges between C₂₈ and C₃₆, consisting of saturated, mono- and diunsaturated components (Table 4). These mycolates possessed predominantly saturated C₁₂ and C₁₄ side-chains R₁, although in one strain (NCTC 3985) major amounts of a monounsaturated C₁₆ side-chain were detected (Table 2). These results are in agreement with previous reports of mycolic acids with carbon ranges of C₂₈ to C₃₂ (Senn *et al.*, 1967; Beaman *et al.*, 1974) and C₂₆ to C₃₄ (Alshamaony *et al.*, 1977) from *C. diphtheriae* strains.

The animal pathogens *C. kutscheri*, '*C. minutissimum*', *C. pseudotuberculosis*, *C. renale* and '*C. ulcerans*' all contained parent mycolic acids with carbon ranges between C₂₆ and C₃₆;

[illegible]

Table 4. (cont.)

[illegible]

No. of carbons in parent mycolic acid	<i>m/e</i>	No. of double bonds	<i>C. segmentum</i>					<i>Corynebacterium</i> spp.					<i>C. ulcerans</i>										<i>C. xerosis</i>				
			C64	D11	D16	D17	D18	C174	C176	C178	C179	C180	H6	H8	C18	C170	C172	C27	C137								
C ₂₈	432	2																									
	434	1																									
	436	0																									
C ₂₉	446	2																									
	448	1	+																+								
C ₃₀	460	2																									
	462	1																									
	464	0																									
C ₃₁	474	2																									
	476	1																									
	478	0																									
C ₃₂	488	2	+																								
	490	1	++																								
	492	0																									
C ₃₃	502	2	+																								
	504	1	+																								
	506	0																									
C ₃₄	516	2	++																								
	518	1	+																								
	520	0																									
C ₃₅	530	2	+																								
	532	1																									
	534	0	+																								
C ₃₆	544	2	++																								
	546	1																									

No. of carbons in parent mycolic acid	<i>m/e</i>	No. of double bonds														
			C795	C228	C905	C790	C903	C260	C113	C188	C901	C1	C104	N146	D8	D9
C ₃₉	586	2		+				+				+		+	++	++
	588	1	+	+	+	+	+		+	+	+			+	+	+
	590	0	+	+	+	+	+		+	+	+				+	+
C ₄₀	598	3												+	+	+
	600	2	+	+	+	+	+	+	+	+	++	+		+	++	+
	602	1	+	+	+	+	+	+	+	+	+	+		+	++	+
	604	0	+	++		+	+	+	+	+	+	+		+	++	+
C ₄₁	612	3														
	614	2		+			+	+		+		+		+	+	+
	616	1	+	+	+	+	+	+	+	+		+		+	+	+
	618	0	+	+	+	+	+	+	+	+		+				
C ₄₂	626	3														
	628	2	+	+	+	+	+	+	+	+	+	+		+	+	+
	630	1	+	++	+	+	+	++	+	+	++	+		+	+	+
	632	0	+	+	+	+	+	+	+	+	+	+				
C ₄₃	640	3														
	642	2	+	+	+	+	+	+	+	+	+	+		+	+	+
	644	1	+	+	+	+	+	+	+	+	+	+		+	+	+
	646	0														
C ₄₄	654	3		+				+				+		+	+	+
	656	2	+	+	+	+	+	+	+	+	+	++		+	+	+
	658	1	+	+	+	+	+	++	+	+	+	++		+	+	+
	660	0						+	+			+		+	+	+
C ₄₅	668	3						+				+				
	670	2	+	+	+	+	+	+	+	+	+	+		+	+	+
	672	1	+	+	+	+	+	++	+	+	+	++		+	+	+
	674	0						+				+				
C ₄₆	682	3						+				+		+	+	+
	684	2	+	+	+	+	+	++	+	+	+	++		+	+	+
	686	1	+	+	+	+	+	++	+	+	+	++		+	+	+
	688	0						+				+		+	+	+
C ₄₇	696	3						+				+				
	698	2						+				+		+	+	+
	700	1						+				+		+	+	+
	702	0						+				+		+	+	+

No. of carbons in parent mycolic acid	m/e	No. of double bonds	<i>A. hydrocarboglutamicus</i> C795	<i>A. paraffinicus</i> C228	<i>A. picolinophilus</i> C905	<i>B. butanicum</i> C790	<i>B. heali</i> C903	<i>B. ketoglutamicum</i> C260	<i>B. paraffinolyticum</i> C113	<i>B. stercoricum</i> C188	<i>C. alkalinum</i> C901	<i>C. fascians</i>					<i>C. hydrocarboactatus</i>		<i>C. rubrum</i> C190	<i>Mycobacterium lacticum</i> C237	<i>Mycobacterium rubrum</i> C239
												C1	C104	N146	D8	D9					
	710	3										+	+	+					+	+	+
	712	2										+	+	+					+	+	+
	714	1										+	+	+					+	+	+
	716	0										+	+	+					+	+	+
	724	3										+	+	+					+	+	+
	726	2										+	+	+					+	+	+
	728	1										+	+	+					+	+	+
	730	0										+	+	+					+	+	+
	738	3										+	+	+					+	+	+
	740	2										+	+	+					+	+	+
	742	1										+	+	+					+	+	+
	744	0										+	+	+					+	+	+
	752	3										+	+	+					+	+	+
	754	2										+	+	+					+	+	+
	756	1										+	+	+					+	+	+
	758	0										+	+	+					+	+	+
	766	3										+	+	+					+	+	+
	768	2										+	+	+					+	+	+
	770	1										+	+	+					+	+	+
	772	0										+	+	+					+	+	+
	780	3										+	+	+					+	+	+
	782	2										+	+	+					+	+	+
	794	3										+	+	+					+	+	+
	796	2										+	+	+					+	+	+
	808	3										+	+	+					+	+	+
	810	2										+	+	+					+	+	+
	812	1										+	+	+					+	+	+
	822	3										+	+	+					+	+	+

Table 6. *Strains having mycolic acids intermediate between Corynebacterium sensu stricto and Rhodococcus types: peaks in the mass spectra of methyl mycolates corresponding to anhydromycolates*

The main component of each series is denoted by +++, any component greater than 50% of the main peak by ++, and all other significant components by +.

No. of carbons in parent mycolic acid	<i>m/e</i>	No. of double bonds	' <i>A. roseoparaffinus</i> ' C112	<i>C. equi</i> C7	<i>C. hoagii</i> C24
C ₃₀	464	0		+	+
C ₃₁	476	1			+
	478	0		+	+
C ₃₂	488	2			+
	490	1		+	+
	492	0		++	++
C ₃₃	502	2	+		+
	504	1	+	+	+
	506	0		+	+
C ₃₄	516	2	+	+	+
	518	1	+	+	+
	520	0		+++	+++
C ₃₅	530	2	+	+	+
	532	1	+	+	+
	534	0		+	+
C ₃₆	544	2	++	+	+
	546	1	++	+	+
	548	0		++	++
C ₃₇	558	2	++	+	+
	560	1	+	+	+
	562	0	+	+	+
C ₃₈	572	2	+++	+	+
	574	1	+	+	+
	576	0		+	+
C ₃₉	586	2	+		

saturated, mono- and diunsaturated components were present in all strains (Table 4). The mycolates of the majority of these strains contained predominantly saturated C₁₄ side-chains, although smaller amounts of saturated C₁₂, C₁₃, C₁₅ and C₁₆ and monounsaturated C₁₄ and C₁₆ side-chains were occasionally detected (Table 2). The present data are in good agreement with the report of the release of a C_{16:0} ester from the mycolates of *C. pseudotuberculosis* on pyrolysis gas chromatography (Lechevalier *et al.*, 1973). Similarly, Diara & Pudles (1959) described a saturated C₃₄ mycolic acid in *C. pseudotuberculosis* which on pyrolysis released a C_{16:0} ester. Yano & Saito (1972), however, reported the presence of mycolic acids in '*C. ulcerans*' having a shorter chain length (C₂₀ to C₃₂) than those observed in the present study.

Strains of the animal-associated species *C. pseudodiphtheriticum* and *C. xerosis* contained mycolic acids with carbon ranges from C₂₈ to C₃₆ (Table 4). Mass spectrometry of the mycolates of *C. pseudodiphtheriticum* showed the predominance of saturated C₁₂H₂₅ side-chains R₁; smaller amounts of saturated C₁₄H₂₉ and monounsaturated C₁₄H₂₇ and C₁₆H₃₁ side-chains were also detected (Table 2). Considerable variation in the composition of the side-chains was observed between strains of *C. xerosis*. The mycolates of *C. xerosis* strain C33 contained major amounts of saturated C₁₂H₂₅ and C₁₄H₂₉ and monounsaturated C₁₄H₂₇ and C₁₆H₃₁ side-chains, whereas those from *C. xerosis* strains C27 and C137 had predominantly saturated C₁₄H₂₉ side-chains (Table 2, Fig. 3). Welby-Giesse *et al.* (1970) found mycolic acids with one double bond in the α -alkyl branch in a strain of *C. pseudodiphtheriticum*. These mycolates had a carbon chain length of C₃₂ to C₃₆ which on

pyrolysis yielded $C_{16:0}$, $C_{16:1}$, $C_{18:0}$ and $C_{18:1}$ esters. In a strain of *C. xerosis*, Alshamaony *et al.* (1977) and Corina & Sesardic (1980) found mycolic acids with carbon ranges of C_{32} to C_{36} and C_{28} to C_{36} , respectively.

The mycolic acids from '*C. flavidum*', '*C. mycetoides*' and '*C. segmentosum*' had carbon ranges from C_{28} to C_{36} (Table 4). Mass spectrometry of the mycolates of '*C. flavidum*' and '*C. segmentosum*' showed the predominance of monounsaturated C_{16} side-chains R_1 ; smaller amounts of saturated C_{12} and C_{14} side-chains were also detected (Table 2). In contrast, the mycolates of '*C. mycetoides*' had major amounts of side-chains with compositions $C_{14}H_{29}$ and $C_{15}H_{31}$; smaller amounts of C_{12} , C_{13} and C_{16} side-chains were also observed in the mass spectrum. The occurrence of major amounts of mycolic acids with side-chains containing odd numbers of carbon atoms was very characteristic and distinguished this taxon from all other coryneform bacteria examined (Table 2).

Strains of the glutamic acid-producing coryneform bacteria – '*Arthrobacter albidus*', '*Brevibacterium ammoniagenes*', '*B. chang-fua*', '*B. divaricatum*', '*B. flavum*', '*B. glutamigenes*', '*B. immariophilum*', '*B. lactofermentum*', '*B. roseum*', '*B. saccharolyticum*', '*B. taipei*', '*Corynebacterium acetoacidophilum*', '*C. callunae*', '*C. glutamicum*', '*C. herculis*', '*C. lilium*', '*C. melassecola*' and '*Microbacterium ammoniaphilum*' – all contained mycolic acids with carbon ranges of C_{28} to C_{36} ; saturated mono- and diunsaturated components were detected. Minor variations in the composition of the side-chains R_1 were observed between individual strains although the general homogeneity exhibited by the mycolic acids of the glutamic acid-producing strains supports their assignment to the genus *Corynebacterium sensu stricto* (Abe *et al.*, 1967; Bousfield & Goodfellow, 1976; Collins *et al.*, 1979a).

The mycolic acids of strains bearing the labels *A. variabilis*, *B. stationis* and '*Microbacterium flavum*' had carbon ranges of C_{28} to C_{36} , composed of saturated, mono- and diunsaturated components. Barksdale *et al.* (1979) recently reported the presence of short-chain mycolic acids in '*Microbacterium flavum*' and reclassified this species in the genus *Corynebacterium*, as *C. flavesceus*. The results of the present study are in accord with this proposal. Keddie & Cure (1977) reported the presence of free mycolic acids in *A. variabilis* and *B. stationis* strains which had chromatographic mobilities compatible with their inclusion in the genus *Rhodococcus*. In contrast, Goodfellow *et al.* (1976) reported that the mycolates obtained from whole-organism methanolysates of *A. variabilis* and *B. stationis* had chromatographic properties similar to those from true corynebacteria. The present data confirm that *A. variabilis* and *B. stationis* have mycolic acids with chain lengths similar to those from true corynebacteria.

The mycolic acids from '*A. roseoparaffinus*', *C. equi* and *C. hoagii* exhibited carbon chain lengths of C_{33} to C_{39} , C_{30} to C_{38} and C_{30} to C_{38} , respectively (Table 6). On mass spectrometry the mycolates of '*A. roseoparaffinus*' released major amounts of $C_{16:0}$ esters, although smaller peaks due to $C_{14:0}$, $C_{15:0}$, $C_{16:1}$, $C_{17:0}$, $C_{18:0}$ and $C_{18:1}$ esters were also detected. In contrast, the mycolates of *C. equi* and *C. hoagii* produced predominant amounts of $C_{14:0}$ and $C_{16:0}$ esters with small amounts of C_{12} , C_{15} , C_{17} and C_{18} esters also detected. The taxonomic position of '*A. roseoparaffinus*', *C. equi* and *C. hoagii* remains problematic, as the chain length of their mycolates overlapped in size with those from representatives of *Corynebacterium sensu stricto* (C_{22} – C_{36}) and *Rhodococcus* (C_{32} – C_{66} ; Minnikin & Goodfellow, 1976, 1980). Other criteria, such as the presence of 10-methyloctadecanoic acid (tuberculostearic acid) in '*A. roseoparaffinus*', *C. equi* and *C. hoagii* (D. E. Minnikin, M. D. Collins & M. Goodfellow, unpublished results), indicate these taxa have a greater affinity with members of the genus *Rhodococcus* than with *Corynebacterium sensu stricto*; however, Keddie & Cure (1977) did not detect free mycolic acids in this strain of '*A. roseoparaffinus*'.

Representative strains of *Caseobacter polymorphus* have mycolic acids with carbon ranges between C_{30} and C_{36} (Table 4). The taxonomic position of this taxon remains unclear, for while the mycolic acid data indicate an affinity to true corynebacteria, the guanine plus cytosine content (65.5–66.9% G + C; Crombach, 1978) suggests a closer relationship to the

rhodococci. Clearly, further comparative studies are required to determine the detailed relationships that exist between caseobacters, corynebacteria and rhodococci.

Mycolic acids with a similar range of molecular weights to those from rhodococci were isolated from a number of differently labelled coryneform strains (Table 5). Thus, '*A. hydrocarboglutamicus*', '*A. paraffineus*', *A. picolinophilus*, '*B. butanicum*' and '*B. healii*' contained mycolic acids with carbon ranges from C_{32} to C_{46} , '*B. ketoglutamicum*' from C_{38} to C_{51} , '*B. paraffinolyticum*' from C_{30} to C_{44} , '*B. sterolicum*' from C_{30} to C_{48} , '*C. alkanum*' from C_{34} to C_{46} , '*C. hydrocarboclastus*' from C_{32} to C_{46} , '*Mycobacterium lacticum*' from C_{42} to C_{56} and '*Mycobacterium rubrum* subsp. *propanicum*' from C_{36} to C_{50} . Mycolic acids with a similar range of molecular weights (C_{32} to C_{44}) have been isolated from a strain of '*Mycobacterium paraffinicum*' (Krasilnikov *et al.*, 1972). The same workers reported the isolation of short-chain mycolates (C_{30} to C_{34}) from a strain labelled '*Mycobacterium lacticum* var. *aliphaticum*' (Krasilnikov *et al.*, 1973). Bousfield & Goodfellow (1976) suggested that '*B. sterolicum*' might be accommodated in the genus *Rhodococcus*; the mycolic acid data support this view. Furthermore, the mycolate data indicate that strains labelled '*A. hydrocarboglutamicus*', '*A. paraffineus*', *A. picolinophilus*, '*B. butanicum*', *B. healii*', '*B. ketoglutamicum*', '*B. paraffinolyticum*', '*C. alkanum*', '*C. hydrocarboclastus*', '*Mycobacterium lacticum*' and '*Mycobacterium rubrum* subsp. *propanicum*' should be reclassified in the genus *Rhodococcus*.

Recently, Goodfellow & Alderson (1977) have reclassified strains of *C. fascians* and *C. rubrum* in the genus *Rhodococcus*. The presence of parent mycolic acids with carbon ranges of C_{38} to C_{52} in both *C. fascians* and *C. rubrum* strains (Table 5) is in accordance with this proposal, as representatives of the genus *Rhodococcus* have mycolates within the range C_{30} to C_{66} (Minnikin & Goodfellow, 1976, 1980).

The results of both the present investigation and earlier studies (Goodfellow *et al.*, 1976; Keddie & Cure, 1977) show that mycolic acid analyses can make an important contribution to coryneform systematics. In particular, the results of the present lipid analyses add to the case for restricting the genus *Corynebacterium* to the human and animal corynebacteria and related saprophytic strains (Barksdale, 1970; Schleifer & Kandler, 1972; Jones, 1975). Thus, the presence of short-chain mycolates (C_{22} to C_{36}) within the animal corynebacteria and related saprophytic strains such as *C. glutamicum* distinguishes these organisms from those in other coryneform taxa such as *Arthrobacter*, *Brevibacterium*, *Cellulomonas*, *Curtobacterium*, *Microbacterium* and *Oerskovia* which lack these characteristic lipids. Members of the genus *Rhodococcus* generally have larger mycolic acids (C_{30} to C_{66}) although a clear distinction between true corynebacteria and rhodococci cannot, at present, be made by analysis of mycolic acids alone.

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