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Thin-layer Chromatographic Analysis of Mycolic Acid and Other Long-chain Components in Whole-organism Methanolysates of Coryneform and Related Taxa

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SUMMARY

Acid methanolysates of strains representing 58 coryneform taxa were examined for mycolic acids and other long-chain constituents by thin-layer chromatography. Mycolic esters were detected in the methanolysates of true corynebacteria but not in those from plant pathogenic bacteria, *Corynebacterium haemolyticum*, *Corynebacterium pyogenes* or from representatives of the genera *Arthrobacter*, *Cellulomonas*, *Curtobacterium*, *Kurthia* or *Oerskovia*. Thin-layer chromatography of whole-organism methanolysates provides a simple method for distinguishing true corynebacteria from coryneforms which do not contain mycolic acids, and from nocardiae and mycobacteria which produce mycolic acids of different mobility. At present the mycolic esters of true corynebacteria cannot be clearly separated from those of some rhodochrous strains.

INTRODUCTION

Bergey's Manual of Determinative Bacteriology currently includes the genera *Arthrobacter*, *Cellulomonas*, *Corynebacterium* and *Kurthia* in the coryneform group and lists *Brevibacterium* and *Microbacterium* as genera *incertae sedis* (Rogosa *et al.*, 1974). The composition of the genus *Corynebacterium* remains unsettled: Jones (1975) would include only *C. diphtheriae* and closely related animal corynebacteria, whereas Rogosa *et al.* (1974) still recommend the inclusion of the plant pathogenic corynebacteria. The results of numerical phenetic studies (Bousfield, 1972; Jones, 1975) and non-numerical studies (Keddie, Leask & Grainger, 1966; Bowie *et al.*, 1972) show that the coryneform bacteria form a heterogeneous group. There is good evidence that chemical markers are of value in the classification and identification of coryneform and related taxa (Cummins, 1962; Yamada & Komagata, 1970; Schleifer & Kandler, 1972).

Mycolic acids, i.e. long-chain 3-hydroxycarboxylic acids having a long alkyl branch on C-2, are lipid components found only in mycobacteria, nocardiae, rhodochrous strains and some corynebacteria (Etémadi, 1967; Maurice, Vacheron & Michel, 1971). The mycolic acids from corynebacteria examined to date have a relatively low molecular weight (20 to 36 carbon atoms) (Pudles & Lederer, 1954; Diara & Pudles, 1959; Etémadi, Gasche & Sifferlen, 1965; Welby-Gieusse, Lanéelle & Asselineau, 1970; Yano & Saito, 1972). Mycolic acids of a similar size have been isolated from strains bearing the labels *Brevibacterium thiogenitalis* (Okazaki *et al.*, 1969), *Arthrobacter paraffineus* (Suzuki *et al.*, 1969) and *Mycobacterium lacticolum* var. *aliphaticum* (Krasilnikov *et al.*, 1973).

Systematic investigations of the mycolic-acid composition of coryneform bacteria have not been performed and it is not known whether these lipids will provide good chemical markers for classification and identification. Analysis by thin-layer chromatography (t.l.c.) of ethanol/diethyl ether (1:1, v/v) extracts of true corynebacteria (Jones, 1975), nocardiae and rhodochrous strains showed that these bacteria contained characteristic lipid components identified as free mycolic acids (Mordarska, Mordarski & Goodfellow, 1972; Goodfellow, 1973; Goodfellow *et al.*, 1973, 1974; Minnikin, Patel & Goodfellow, 1974; Alshamaony *et al.*, 1976*a*, *b*). A more convenient procedure, involving t.l.c. of acid methanolysates of dry bacteria, has been developed for analysing the content of mycolic acid and other long-chain constituents (Minnikin, Alshamaony & Goodfellow, 1975). We have used this whole-organism methanolysis technique to examine 122 strains representing 58 coryneform taxa.

METHODS

Cultures. The test strains are listed in Table 1. *Corynebacterium haemolyticum* and *C. pyogenes* cultures were maintained on brain–heart infusion agar (Oxoid); other strains were maintained on Dorset Egg and Loeffler serum slopes (Cowan, 1974).

Cultivation. Corynebacterium haemolyticum and *C. pyogenes* were grown in brain–heart infusion broth (Oxoid) in stationary culture for 4 days at 37 °C, the animal corynebacteria were grown in shake flasks of nutrient broth (Oxoid) supplemented with 1 % (w/v) Tween 80 for 2 days at 37 °C, and the other were strains grown in shake flasks of nutrient broth for 3 days at 30 °C. *Corynebacterium* sp. KD was grown on Mueller–Hinton Agar (Oxoid) supplemented with 0·1 % (w/v) cysteine.

Cultivated organisms were killed with 1 % (v/v) formaldehyde, harvested by centrifuging, washed with distilled water and freeze-dried.

Whole-organism methanolysis and thin-layer chromatography. Dried organisms were examined using the acid methanolysis and t.l.c. procedure described by Minnikin *et al* (1975).

RESULTS AND DISCUSSION

The patterns obtained by chromatography of methanolysates of coryneform bacteria, and of the reference Nocardia, Mycobacterium and rhodochrous strains, are shown in Fig. 1. The identity of spots corresponding to mycolic esters was confirmed by washing the developed chromatogram with a mixture of methanol/water (5:2, v/v), which removed all spots except those corresponding to the mycolic esters (Minnikin *et al.*, 1975). The spots on the chromatograms having R_F values greater than 0·6 are attributable to the methyl esters of non-hydroxylated long-chain fatty acids. On the basis of the results the strains were clustered into six groups: A, B, C, D, E and F (see Table 1, Fig. 1).

Mycolic esters were detected in methanolysates of Nocardia, rhodochrous strains, certain Corynebacterium strains and in the single strain of Mycobacterium examined. The extract from *Mycobacterium avium* (Table 1, Group E; Fig. 1) gave a multispot pattern in accordance with previous studies (Minnikin *et al.*, 1975; Etémadi, 1967); single spots were obtained for the mycolates from the other genera included in Groups A, B, C and D. The mycolate from the representative strains of Nocardia (Group D) had a relatively high mobility (Fig. 1) in comparison with mycolates from Group C strains, which include an authentic representative of the '*rhodochrous*' complex. The esters of the mycolic acids from strains in Group A, which includes type and authentic strains of the genus *Corynebacterium*, had the lowest mobility (Fig. 1). Representatives of *C. equi* and

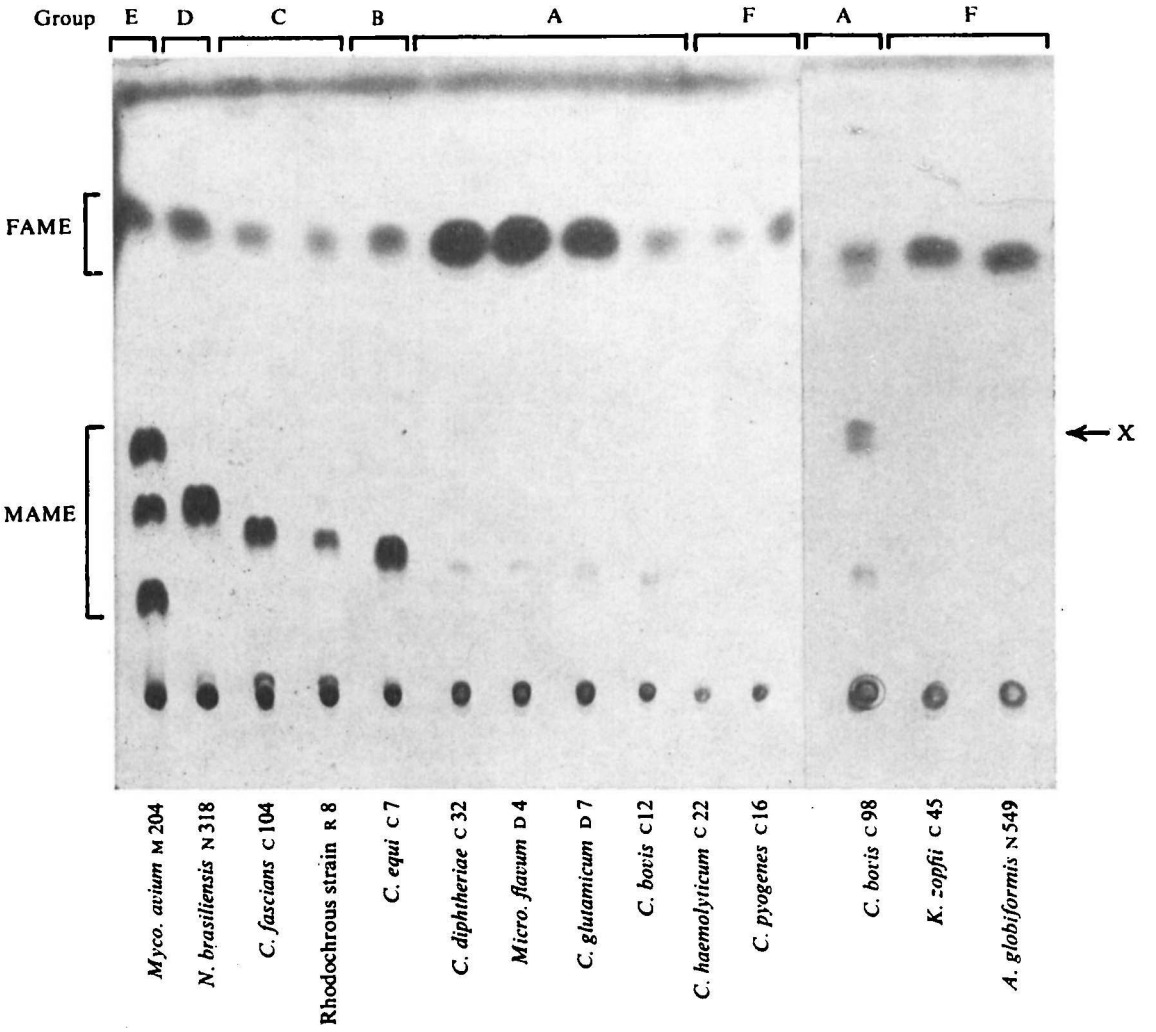

Fig. 1. Thin-layer chromatography of whole-organism methanolysates of selected bacteria. FAME, fatty acid methyl esters; MAME, mycolic acid methyl esters; X, unknown components.

Brevibacterium paraffinolyticum (Group B) gave mycolates whose mobilities were difficult to distinguish clearly from those of representatives of Groups A and C (Fig. 1). The spots corresponding to mycolates in extracts of Group A were always relatively low in intensity compared with the spots derived from the simple non-hydroxylated fatty acids (Fig. 1); whereas the mycolic ester spots in methanolysates of Groups C and D and the strains of *C. equi* (Group B) were of much greater relative intensity. Mycolic acids were not found in the other coryneform taxa investigated (Group F).

Methanolysates of four strains included in Group A contained additional components (X) which gave spots on t.l.c. having mobilities intermediate between the mycolic and non-hydroxylated esters, e.g. *C. bovis* C98 (Fig. 1). Two other strains of *C. bovis* (C97 and C100) and a single representative of *C. xerosis* (C33) gave similar patterns, the only difference being that single spots were seen in contrast to the double spot for methanolysates of

Table 1. *Grouping of strains based on chromatographic analysis (see Fig. 1)*

Strain	No.	Strain designation/source†
Group A		
Arthrobacter albidus	1	D6 (NCIB10266)
A. roseoparaffineus	1	C112 (NCIB10700)
A. variabilis	1	D2 (NCIB9455)
Brevibacterium ammoniagenes	1	C80 (NCIB8143)
B. divaricatum	1	D3 (NCIB9379)
B. flavum	1	C81 (NCIB9565)
B. roseum	1	C82 (NCIB9564)
B. stationis	1	*C41 (ATCC14403)
Corynebacterium bovis	10	*C12 (NCTC3224); C95, C96, C97 (NIRD1689, 1718, 1928); C98, C99, C100, C101, C102, C103 (J. E. Schreeve, Central Veterinary Laboratory, Weybridge, Surrey, DB223/75, DB132/75, DB210/75, DB30/75, DB94/75, 120B)
C. diphtheriae	3	C13 (NCTC3985); C14 (NCTC3987); *C32 (NCTC3984)
C. flavidum	1	C35 (NCTC764)
C. glutamicum	1	D7 (NCIB10025)
C. herculis	1	*C85 (NCIB9694)
C. hoagi	1	C24 (NCTC10673)
C. minutissimum	2	D24B (NCTC10285); *D24 (NCTC10288)
C. pseudodiphtheriticum	2	C19 (NCTC231); C73 (D. Jones, Microbial Systematics Unit, University of Leicester, C10)
C. pseudotuberculosis	3	C15 (NCTC3450); H5, H7 (P. Maximescu, Dr I. Cantacuzino Institute, Bucharest, Rumania, 992, 993)
C. renale	5	*C17 (NCTC7448); H1, H2, H3, H4 (R. Yanagawa, Hokkaido University, Sapporo, Japan, 43, 45, 46, 42)
C. segmentosum	1	C64 (NCTC934)
C. ulcerans	3	C18 (NCTC7910); H6, H8 (P. Maximescu, 896, 985)
C. xerosis	2	C27 (NCTC7238), C33 (NCTC8755)
'*Corynebacterium*' sp.	9	D10 to D18 (I. J. Bousfield, NCIB, SN65, SN66, SN71, SN93, SN153, SN135, SN123, SN149, SN130)
Microbacterium flavum	1	*D4 (NCIB8707)
Group B		
B. paraffinolyticum	1	C113 (NCIB11160)
C. equi	9	*C7 (NCTC1621); C56 (NCTC5649); C57 (NCTC5650); C58 (NCTC4219); D19, D20, D21, D22, D23 (H. R. Carne, Pathology Department, University of Cambridge, 20343, Jeffcott 1, 149, 1499, Jeffcott 2)
Group C		
C. fascians	2	*C39 (ATCC12974); C104 (NCPPB1488)
C. hydrocarboclastus	2	*D8, D9 (K. Komagata, Ajinomoto Co., Kawasaki, Japan, AJ1386, AJ1379)
Rhodochrous strain	1	R8 (ATCC4276)
Group D		
Nocardia brasiliensis	1	*N318 (ATCC19296)
Group E		
Mycobacterium avium	1	M204 (Central Veterinary Laboratory, Weybridge, Surrey, D4)

Table 1. (*cont.*)

Strain	No.	Strain designation source†
Group F		
A. globiformis	1	*N549 (NCIB8707)
A. simplex	1	*N295 (NCIB8929)
Bacterium eurydice	2	C49, C50 (D. Jones, C207, C208)
Brevibacterium imperiale	1	*C43 (ATCC8365)
B. linens	3	C40 (ATCC9174); C83 (NCIB9909); D1 (NCIB8546)
B. sulphureum	1	C79 (NCIB10355)
Cellulomonas flavigena	1	*CIII (NCIB8073)
Corynebacterium acnes	1	P1 (ATCC6921)
C. aquaticum	1	*C84 (NCIB9460)
C. barkeri	1	*C8 (NCIB9658)
C. betae	1	C3 (NCPPB363)
C. flaccumfaciens	1	C9 (NCPPB559)
C. haemolyticum	2	*C22 (NCTC8452); C23 (NCTC9998)
C. ilicis	1	*C2 (ATCC14264)
C. insidiosum	1	C10 (NCPPB83)
C. michiganense	1	C8 (NCPPB1468)
C. nebraskensis	3	*H11, H12, H13 (A. Vidaver, University of Nebraska, Lincoln, Nebraska, U.S.A., Fur-1, Bennett Goth, 721-s)
C. okanaganae	1	*H10 (P. Luthy, Mikrobiologisches Institut, Zürich, Switzerland, B4405)
C. poinsettiae	1	C11 (NCPPB844)
C. pyogenes	3	*C16 (NCTC5224); D25 (NCTC6488); D26 (NCTC10513)
C. rathayi	1	C4 (NCPPB797)
'*Corynebacterium*' sp.	1	H14 (G. L. Bullock, Kearneysville, West Virginia, U.S.A., strain KD)
'Cheese coryneform bacteria'	5	D24, D25, D26, D27, D28 (M. E. Sharpe, NIRD, CMD1, CMD3, C4, R6, B4)
Curtobacterium albidum	1	C92 (NCIB11030)
Curtobacterium citreum	1	*C93 (NCIB10702)
Curtobacterium luteum	1	*C94 (NCIB11029)
Kurthia zopfii	5	C37, C38, C72, C77 (D. Jones, C5, C6, C7, C205); *C45 (NCTC404)
Microbacterium lacticum	2	*C90 (NCIB8540); C91 (NCIB8541)
Micro. thermosphactum	5	C105, C106, C107, C108, *C109 (D. Jones, C1, C2, C3, C4, C20)
Mycobacterium flavum	1	D5 (NCIB10071)
Oerskovia turbata	1	*CII0 (NCIB10587)

* Type strain.

† ATCC, American Type Culture Collection, Rockville, Maryland, U.S.A.; NCIB, National Collection of Industrial Bacteria, Aberdeen; NCPPB, National Collection of Plant Pathogenic Bacteria, Harpenden; NCTC, National Collection of Type Cultures, London; NIRD, National Institute for Research in Dairying, Shinfield, Reading; C, D, H, N, P and R, laboratory numbers.

C. bovis C98 (Fig. 1). The nature of these additional components remains to be determined; they can, however, be distinguished from mycolic acids by their mobility on t.l.c. using methanol/water (5:2, v/v) (Minnikin *et al.*, 1975). Long-chain alcohols, such as hentriacontan-16-ol and nocardols found previously in certain strains of nocardioform bacteria (Bordet & Michel, 1964; 1969; Lanéelle, Asselineau & Castelnuovo, 1965), would be expected to have chromatographic mobilities similar to those of the unknown components.

Our data correlate well with the numerical groupings obtained by Bousfield (1972) and Jones (1975), many strains being common to all three studies. If, as seems advisable,

the genus *Corynebacterium* is restricted to the animal corynebacteria and related taxa such as *C. glutamicum* and strains presently labelled *Arthrobacter albidus*, *A. roseoparaffineus*, *A. variabilis*, *Brevibacterium ammoniagenes*, *B. divaricatum*, *B. flavum*, *B. roseum*, *B. stationis* and *Microbacterium flavum*, then investigations of mycolic-acid composition should be of value in the recognition of such strains. In this connection, a number of strains (Group A, D10 to D18) isolated from marine fish and provisionally identified as *Corynebacterium* species (Bousfield, Gunawardana & Noble, 1976) produced, after methanolysis, mycolic acid esters having the same t.l.c. mobility as those from established species of *Corynebacterium*. On the other hand, some methanethiol-producing 'cheese coryneform bacteria' (Sharpe, Law & Phillips, 1976) (Group F, D24 to D28) and the '*Corynebacterium*' sp. KD, pathogenic for trout and salmon (Ordal & Earp, 1956) (Group F, H14), did not contain mycolic acids.

The finding that strains labelled *C. fascians* and *C. hydrocarboclastus* produced mycolic acid esters with an R_F similar to that of many rhodochrous strains supports the case for reclassifying these taxa in the '*rhodochrous*' complex (Gordon, 1966; Bousfield, 1972; Komura, Komagata & Mitsugi, 1973; Jones, 1975). The taxonomic status of *C. equi* is still debatable for while Bousfield (1972) recovered the type strain in the same phenon as the animal corynebacteria, others have classified it in the '*rhodochrous*' complex (Goodfellow *et al.*, 1974; Jones, 1975) and in the present study representatives of *C. equi* were not clearly distinguished from bacteria in either of these groupings. Jones (1975) also recovered *B. ammoniagenes* and *B. stationis* in the same phenon as *C. equi*, but in the present study these brevibacteria gave mycolates whose mobilities were similar to those of strains placed in Group A.

Corynebacterium haemolyticum, *C. pyogenes*, the remaining plant pathogenic corynebacteria, and saprophytic strains of taxa such as *C. aquaticum* did not contain mycolic acids and can, therefore, be clearly separated from the true corynebacteria (Jones, 1975).

Thin-layer chromatography of whole-organism methanolysates thus provides a simple method for distinguishing true corynebacteria from a host of other coryneforms which do not contain mycolic acids. Analysis of mycolic acid methyl esters by t.l.c. does not, however, presently allow clear distinctions to be made between representatives of true corynebacteria, *C. equi*, *B. paraffinolyticum* and the '*rhodochrous*' complex.

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