Fatty Acid, Isoprenoid Quinone and Polar Lipid Composition in the Classification of *Curtobacterium* and Related Taxa

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Strains representing the taxa *Curtobacterium*, *Brevibacterium saperdae*, *B. testaceum*, *Corynebacterium betae*, *Cor. nebraskense* and *Cor. oortii* were degraded by acid methanolysis and the non-hydroxylated fatty acid esters released were examined by thin-layer and gas-liquid chromatography. The major fatty acids in all strains were 12-methyltetradecanoic (*anteiso* C₁₅) and 14-methylhexadecanoic (*anteiso* C₁₇) acids which occurred together with other *anteiso*, *iso* and straight-chain acids. Polar lipids of the test strains were examined by two-dimensional thin-layer chromatography. All organisms possessed very characteristic polar lipid patterns consisting of diphosphatidylglycerol, phosphatidylglycerol and a number of uncharacterized glycolipids. The menaquinone components of the test strains facilitated their division into two groups containing, respectively, nine isoprene units (viz. *Curtobacterium citreum*, *Curt. luteum*, *Corynebacterium betae*, *Cor. flaccumfaciens*, *Cor. oortii*, *Cor. poinsettiae* and *Cor. nebraskense*) and eleven and twelve isoprene units (viz. *Brevibacterium saperdae*, *B. testaceum*). The results of the present study indicate that the genus *Curtobacterium* as presently recognized is a heterogeneous taxon containing two distinct centres of variation.

INTRODUCTION

The genus *Curtobacterium* was proposed by Yamada & Komagata (1972) to accommodate certain 'motile brevibacteria' and the phytopathogens *Corynebacterium flaccumfaciens* and *Cor. poinsettiae*. Subsequent studies have indicated that *Cor. betae* should also be included in the genus (Jones, 1975; Keddie & Cure, 1977). The main distinguishing features for curtobacteria are the presence of ornithine in the cell wall, DNA containing 66 to 71 mol % G + C, and slow and weak acid production from some carbohydrates. Additional criteria are required to tighten the genus description and to help separate *Curtobacterium* from other ornithine-containing coryneform taxa such as *Cellulomonas*.

Recently, lipid composition has been found to be of value in the classification and identification of coryneform bacteria (Minnikin *et al.*, 1978). The most productive lipid studies on curtobacteria have involved the analyses of isoprenoid quinones (Yamada *et al.*, 1976; Collins *et al.*, 1979). The fatty acid and polar lipid compositions of curtobacteria have received less attention (Tadayon & Carroll, 1971; Komura *et al.*, 1975) and it is not known

whether they are of value in the classification of the group. In the present study, the isoprenoid quinones, polar lipids and fatty acid profiles of representative strains of *Curtobacterium*, *Brevibacterium saperdae* (Lysenko, 1959), *B. testaceum* (Komagata *et al.*, 1964), *Cor. nebraskense* (Vidaver & Mandel, 1974) and *Cor. oortii* (Saaltink & Mass Geesteranus, 1969) were examined in an attempt to clarify their taxonomy.

METHODS

Cultures and cultivation. The test strains listed in Table 1 were maintained on Dorset Egg (Cowan, 1974) and nutrient agar (Oxoid no. 2) slopes at room temperature, and cultivated in 400 ml nutrient broth (Oxoid no. 2) at 30 °C for 2 d. The flasks were aerated by shaking in an orbital incubator at 150 rev. min⁻¹. Cultures were checked for purity at maximum growth, harvested by centrifugation (5000 g), washed with distilled water and freeze-dried.

Extraction and analysis of fatty acid methyl esters. Dried organisms (50 mg) were examined using the acid methanolysis and thin-layer chromatography (t.l.c.) procedure described by Minnikin *et al.* (1975). Fatty acid methyl esters were purified by preparative t.l.c. on Merck silica gel PF₂₅₄₊₃₆₆ plates (1 mm thick) using petroleum ether (b.p. 60 to 80 °C)/diethyl ether (85:15, v/v) as developing solvent. The fatty acid methyl esters were located by viewing under ultraviolet light (366 nm) and eluted from the gel with chloroform. For the analysis of the fatty acid methyl esters, a Perkin-Elmer F11 flame-ionization gas chromatograph fitted with stainless steel columns (4 mm o.d.) was used, with nitrogen as carrier gas. Initial analyses were performed using both non-polar OV-1 (2 m, 2.5% OV-1 silicone on 80 to 100 mesh Chromosorb G AW-DMCS, 180 °C; Phase Separations) and polar (2 m, 3% Silar 5CP on 80 to 100 mesh Gas Chrom Q, 160 °C; Applied Science Laboratories) columns. Resolution of mixtures of *iso*- and *anteiso*-methyl branched acids was achieved using a 6 m column packed with 10% Silar 10C on 100 to 120 mesh Gas Chrom Q (Applied Science Laboratories) at 210 °C. The identity of individual esters was established by comparison of the retention times with those of standard mixtures of straight-chain and monounsaturated esters and a branched-chain standard mixture (BC Mix-L; Applied Science Laboratories). Assignment of fatty acid esters was also assisted by calculation of equivalent chain lengths (Miwa *et al.*, 1960) for each stationary phase employed. The relative proportions of the fatty acid esters were estimated by calculating the product of the retention time and peak height for each component and taking percentages of the sum of the products for all the components (Kates, 1972).

Extraction and analysis of polar lipids. Dry organisms (100 mg) were extracted using a modification (Card, 1973; Minnikin *et al.*, 1979) of the procedure of Bligh & Dyer (1959). The polar lipids were analysed by two-dimensional t.l.c. on Merck silica gel H plates (0.5 mm thick) impregnated with sodium acetate (Minnikin & Abdolrahimzadeh, 1971; Minnikin *et al.*, 1979). Chromatograms were developed in the first dimension with chloroform/methanol/water (65:25:4, by vol.) and in the second with chloroform/acetic acid/methanol/water (80:15:12:4, by vol.). Lipids were revealed by spraying the chromatograms with 40% H₂SO₄ followed by charring at 180 °C. Specific spray reagents for lipid phosphate (Dittmer & Lester, 1964), α -glycols (periodate-Schiff) (Shaw, 1968), sugars (α -naphthol, and anisaldehyde/H₂SO₄) (Jacin & Mishkin, 1965; Stahl & Kaltenbach, 1961) and free amino groups (ninhydrin in water-saturated butanol) were also used.

Extraction, purification and analysis of isoprenoid quinones. Dried organisms (150 mg) were examined using the procedure described by Collins *et al.* (1977). Purified isoprenoid quinones were further examined by reverse phase partition chromatography using Merck HPTLC RP-18F₂₅₄ reverse phase thin-layer plates (10 × 10 cm) and a polar developing mixture of acetone/water (99:1, v/v) (Collins *et al.*, 1980). Separated components were detected under ultraviolet light (254 nm). Ultraviolet spectra of the quinones were recorded in iso-octane on a Perkin-Elmer (model 137UV) spectrophotometer. Mass spectra of the isoprenoid quinones were recorded on an AEI MS9 instrument using a direct insertion probe, an ionizing voltage of 70 eV and a temperature range of 200 to 220 °C.

RESULTS AND DISCUSSION

Thin-layer chromatographic analysis of the whole-organism methanolysates of the test strains revealed single spots corresponding to non-hydroxylated long-chain fatty acid methyl esters (Minnikin *et al.*, 1975, 1978). The non-hydroxylated fatty acids of the test strains were primarily *anteiso*- and *iso*-methyl branched-chain acids (90.5 to 99.4%) although straight-chain acids (0.6 to 9.5%) were also present in small amounts (Table 1). The major fatty acids in all strains were 14-methylhexadecanoic (*anteiso* C₁₇) and 12-methyltetradecanoic (*anteiso*

Table 1. Percentage fatty acid composition of the test strains

Equivalent chain lengths on respective g.l.c. columns*	OV-1 Silar 5CP (Silar 10C	13-7	14-0	14-0	14-8	14-7	15-0	15-8	16-0	16-8	17-0	17-8	18-0	Fatty acid total	
		13-5	14-0	14-0	14-7	14-7	15-0	15-7	16-0	16-7	17-0	17-7	18-0		
		13-4	14-0	14-0	14-4	14-4	15-0	15-4	16-0	16-4	17-0	17-4	18-0		
Probable identity†		i-14	14-0	14-0	i-15	ai-15	15-0	i-16	16-0	i-17	ai-17	i-18	18-0	ai	
Lab. no.	Strain designation	Source													
C262	<i>Brevibacterium saperdae</i>	1-9	0-3		6-3	29-4	tr	21-6	6-7	7-8	21-6	1-4	1-1	9-5	51-0
C263	<i>B. testaceum</i>	2-4	0-2		4-8	27-0	tr	24-9	2-9	5-6	29-0	1-2	0-5	4-8	56-0
C3	<i>Corynebacterium betae</i>	0-2	0-1		3-2	32-4	0-5	11-9	1-4	1-5	48-4	0-4	—	2-4	80-8
C258	<i>Cor. betae</i>	0-3	0-1		3-4	32-6	0-1	11-8	1-5	1-3	48-9	tr	—	1-7	81-5
C9	<i>Cor. flaccumfaciens</i>	0-2	0-1		3-2	32-6	0-3	11-8	1-5	1-4	48-7	0-2	—	2-1	81-3
C115	<i>Cor. flaccumfaciens</i>	0-3	0-1		3-6	32-4	0-1	11-8	1-3	1-5	48-7	0-2	—	1-7	81-1
C690	<i>Cor. flaccumfaciens</i> subsp. <i>aurantiacum</i>	0-2	0-1		4-7	31-6	tr	11-6	0-8	2-8	47-8	0-4	—	1-3	79-4
H11	<i>Cor. nebraskense</i>	0-1	—		0-3	33-0	tr	11-7	1-9	1-2	51-2	0-3	tr	2-2	84-2
H12	<i>Cor. nebraskense</i>	0-1	—		0-4	34-8	tr	12-2	1-5	0-6	50-0	0-2	tr	1-7	84-8
C617	<i>Cor. oortii</i>	0-4	0-1		2-7	32-8	0-2	12-0	0-9	1-1	49-8	tr	—	1-2	82-6
C618	<i>Cor. oortii</i>	0-6	tr		4-2	32-1	0-3	12-3	1-1	1-5	46-9	1-0	—	2-4	79-0
C11	<i>Cor. poinsettiae</i>	0-3	0-1		3-8	32-2	0-1	11-7	1-4	1-5	48-8	0-1	—	tr	81-0
C93	<i>Curtobacterium citreum</i>	0-8	tr		4-5	28-1	0-3	20-5	1-5	2-7	40-5	0-6	0-5	2-4	68-6
C94	<i>Curt. luteum</i>	5-9	tr		5-6	23-2	0-2	26-0	0-4	3-3	32-7	tr	2-7	tr	55-9

tr, Trace.

* According to Miwa *et al.* (1960).† Abbreviations for fatty acids: 15:0 denotes straight-chain pentadecanoic acid; i-15 denotes the *iso*-acid 13-methyltetradecanoic acid; ai-15 denotes the *anteiso*-acid 12-methyltetradecanoic acid; etc.

Table 2. *Peaks corresponding to molecular ions (M^+) in the mass spectra of menaquinones isolated from the test strains*

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 +.

Menaquinone isoprenologue <i>m/e</i>	MK-8 716	MK-9 784	MK-10 852	MK-11 920	MK-12 988	MK-13 ³ 1056
<i>Brevibacterium saperdae</i> C262	+	+	+	++*	+++	+
<i>B. testaceum</i> C263	+	+	+	+++	++	+
<i>Corynebacterium betae</i> C3	+	+++	—	—	—	—
<i>Cor. betae</i> C258	+	+++	—	—	—	—
<i>Cor. flaccumfaciens</i> C9	+	+++	—	—	—	—
<i>Cor. flaccumfaciens</i> C115	+	+++	—	—	—	—
<i>Cor. flaccumfaciens</i> C690	+	+++	—	—	—	—
<i>Cor. nebraskense</i> H11	+	+++	+	—	—	—
<i>Cor. nebraskense</i> H12	+	+++	+	—	—	—
<i>Cor. oortii</i> C617	+	+++	—	—	—	—
<i>Cor. oortii</i> C618	+	+++	—	—	—	—
<i>Cor. poinsettiae</i> C11	+	+++	—	—	—	—
<i>Curtobacterium citreum</i> C93	+	+++	—	—	—	—
<i>Curt. luteum</i> C94	+	+++	—	—	—	—

* Relative proportions of the isoprenologues were confirmed by reverse phase partition t.l.c.

C_{15}) acids, with 14-methylpentadecanoic (*iso* C_{16}) acid also present in fairly high proportions. The amount of *iso* C_{16} varied considerably among the test strains; substantially higher levels were found in *Brevibacterium saperdae*, *B. testaceum*, *Curtobacterium citreum* and *Curt. luteum* (20.5 to 26.0%) than in the 'phytopathogenic corynebacteria' (11.6 to 12.3%) (Table 1). The results of the present study are in accord with the previous report of high proportions of *iso*- and *anteiso*-methyl branched acids in *Corynebacterium poinsettiae* (Tadayon & Carroll, 1971) and support the separation of *Cor. betae*, *Cor. flaccumfaciens*, *Cor. flaccumfaciens* subsp. *aurantiacum*, *Cor. nebraskense* and *Cor. oortii* from true corynebacteria which contain predominantly straight-chain and monounsaturated fatty acids (Asselineau, 1961; Blaschy & Zimmermann, 1971; Alshamaony *et al.*, 1977; Minnikin *et al.*, 1978). Fatty acid analyses may also prove to be a reliable way of distinguishing curtobacteria from cellulomonads as the latter contain *anteiso* C_{15} as the major acid together with high proportions of straight-chain fatty acids (Minnikin *et al.*, 1979).

Components that chromatographed with vitamin K were the only isoprenoid quinones detected in the test strains. On examination by ultraviolet spectroscopy the isoprenoid quinones displayed absorption maxima at 242, 248, 260, 270 and 326 nm in accordance with published data for menaquinones (Dunphy & Brodie, 1971). The mass spectra of all the menaquinone samples showed intense peaks at *m/e* 187 and 225 derived from the naphthoquinone nucleus, as expected from published data (Azerad & Cyrot-Pelletier, 1973). The mass spectra in the high mass region contained strong peaks corresponding to molecular ions (M^+), with smaller peaks at $M^+ - 15$ corresponding to the loss of a methyl group from the molecular ion. Details of the mass spectral analyses of the menaquinones are shown in Table 2. The menaquinone composition of the test strains facilitated their division into two groups. Strains of *Cor. betae*, *Cor. flaccumfaciens*, *Cor. flaccumfaciens* subsp. *aurantiacum*, *Cor. nebraskense*, *Cor. oortii*, *Cor. poinsettiae*, *Curt. citreum* and *Curt. luteum* contained unsaturated menaquinones with nine isoprene units (abbreviated as MK-9) as the predominant isoprenologue, a result in accord with earlier studies (Collins *et al.*, 1979, 1980). *Brevibacterium* (*Curtobacterium*) *testaceum*, however, contained MK-11 as the major isoprenologue together with substantial amounts of MK-12, thereby confirming the earlier work of Yamada *et al.* (1976). The single strain of *B. saperdae* examined also contained major amounts of MK-11 and MK-12 although the latter component predominated (Table 2).

Fig. 1. Two-dimensional thin-layer chromatograms of polar lipids from *Corynebacterium betae*, *Corynebacterium flaccumfaciens* and *Corynebacterium poinsettiae*. Chloroform/methanol/water (65:25:4, by vol.) was used in the first dimension, and chloroform/acetic acid/methanol/water (80:15:12:4, by vol.) was used in the second dimension. Abbreviations: DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; G, glycolipid.

Fig. 2. Two-dimensional thin-layer chromatograms of polar lipids from *Corynebacterium oortii*, *Corynebacterium nebraskense*, *Curtobacterium citreum* and *Curtobacterium luteum*. Chloroform/methanol/water (65:25:4, by vol.) was used in the first dimension, and chloroform/acetic acid/methanol/water (80:15:12:4, by vol.) was used in the second dimension. Abbreviations: DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; G, glycolipid.

Fig. 3. Two-dimensional thin-layer chromatograms of polar lipids from *Brevibacterium saperdae* and *Brevibacterium testaceum*. Chloroform/methanol/water (65:25:4, by vol.) was used in the first dimension, and chloroform/acetic acid/methanol/water (80:15:12:4, vol.) was used in the second dimension. Abbreviations: DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; G, glycolipid.

Two distinct polar lipid patterns were also found in the test strains (Fig. 1, 2, 3). Diphosphatidylglycerol (DPG) and phosphatidylglycerol (PG) were readily identified in all extracts by their chromatographic behaviour and staining properties. A number of other characteristic lipids, which gave negative reactions to the lipid phosphorus and ninhydrin sprays but positive reactions to the periodate-Schiff and α -naphthol reagents, were also detected in all of the strains. These unidentified glycolipids (G) gave green coloration with the anisaldehyde/ H_2SO_4 reagent indicating the possible presence of mannose residues within these lipids. *Curtobacterium citreum*, *Curt. luteum* and the 'phytopathogenic corynebacteria' contained two or more of these unidentified glycolipids (Fig. 1, 2) whereas *B. saperdae* and *B. testaceum* contained only a single glycolipid (Fig. 3). The only previous investigation of the polar lipid composition of curtobacteria and related strains was by Komura *et al.* (1975), who reported that *Curt. albidum*, *Curt. citreum*, *Cor. flaccumfaciens* and *Cor. poinsettiae* possess DPG, PG, phosphatidylinositol (PI) and an uncharacterized phosphatidylinositol mannoside (PIM). The detection of PI and PIM is at variance with the present results (Fig. 1) although Komura *et al.* (1975) did not investigate the possible presence of glycolipids in their strains.

The results of the present study support the inclusion of *Cor. flaccumfaciens* and *Cor. poinsettiae* in the genus *Curtobacterium* (Yamada & Komagata, 1972) and suggest *Cor. betae* and *Cor. oortii* should also be added to the group. The recently described phytopathogen *Cor. nebraskense* (Vidaver & Mandel, 1974) also has a lipid composition remarkably similar to that of curtobacteria. The numerical phenetic study of Dye & Kemp (1977), however, indicated that *Cor. nebraskense* was quite distinct from the ornithine-containing taxa *Cor. betae*, *Cor. flaccumfaciens* and *Cor. poinsettiae* but shared a high affinity with *Cor. michiganense* and other diaminobutyric acid-containing taxa. Further comparative studies, including cell wall analyses, between *Cor. nebraskense*, curtobacteria and diaminobutyric acid-containing coryneform taxa are necessary to clarify the true taxonomic position of *Cor. nebraskense*.

Yamada & Komagata (1972) proposed *B. saperdae* and *B. testaceum* should also be included in the genus *Curtobacterium*. The results of the present study, however, are at variance with this proposal. Curtobacteria should probably be restricted to those taxa

having a peptidoglycan based on D-ornithine (variation B2 β) (Schleifer & Kandler, 1972) and totally unsaturated menaquinones with nine isoprene units (Table 2). *Brevibacterium saperdae* and *B. testaceum* contain distinctively larger menaquinones (MK-11, MK-12) and these strains should be compared further with other ornithine-containing coryneforms (*Arthrobacter flavescens*, *Corynebacterium barkeri*, *Microbacterium liquefaciens*, etc.) of uncertain taxonomic position.

It can be concluded on the basis of lipid composition that the genus *Curtobacterium* as presently constituted represents a heterogeneous taxon.

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