Taxonomic Significance of Cellular Fatty Acid Composition in Some Coryneform Bacteria

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A total of 76 strains of coryneform bacteria belonging to the genera *Arthrobacter*, *Brevibacterium*, *Caseobacter*, *Cellulomonas*, *Corynebacterium*, and *Curtobacterium* were divided into four groups on the basis of their cellular fatty acid compositions. Cells with saturated and monounsaturated straight-chain fatty acids were designated type I. Strains in this group had *meso*-diaminopimelic acid and arabinogalactan in their cell walls. In some strains, 10-methyl fatty acids were found. Type I was divided into six subtypes based on fatty acid composition. Type II cells contained iso-anteiso acids and were found in 43 strains of *Arthrobacter*, *Brevibacterium*, *Cellulomonas*, *Curtobacterium*, and coryneform bacteria with diaminobutyric acid-peptidoglycan. Small differences in fatty acid composition were found among the strains of this type, and the fatty acid compositions of type II strains varied remarkably depending on the media used. Type III strains were characterized by the presence of ω -cyclohexyl fatty acids. In two strains of *Curtobacterium pusillum*, approximately 60% of the cellular fatty acid was ω -cyclohexyl undecanoic acid. Type IV strains had highly complex patterns of iso, anteiso, normal, saturated, unsaturated, 10-methyl, and 2-hydroxy fatty acids. Five strains of *Arthrobacter simplex*, *Arthrobacter tumescens*, and “*Brevibacterium lipolyticum*” possessed this type of fatty acid composition.

Difficulties have been encountered in identifying and classifying coryneform bacteria belonging to the genera *Arthrobacter*, *Brevibacterium*, *Caseobacter*, *Cellulomonas*, *Corynebacterium*, *Curtobacterium*, and *Microbacterium* (21, 35, 49). More recently, data on the deoxyribonucleic acid (DNA) base compositions (2, 8, 38, 48), DNA-DNA homologies (9, 13, 39, 40, 42), peptidoglycan types in the cell walls (12, 22, 23, 36, 47), phospholipid compositions (4, 5, 25, 29, 31), isoprenoid quinones (3–5, 7, 50), and acyl types in the cell walls (44, 45) of these organisms have been of great value in more precise characterizations of many coryneform bacteria (21, 49). Information regarding cellular fatty acid composition has been useful in bacterial taxonomy (16–19, 30, 34, 46) and also has been applied to the taxonomy of some coryneform bacteria (1, 2, 4–6, 26, 29, 30, 31), but the number of coryneform strains heretofore examined has been rather limited. Recently, Suzuki et al. (43) found unusual fatty acids (ω -cyclohexyl fatty acids) in two strains of *Curtobacterium pusillum*.

In this study we examined the cellular fatty acid compositions of coryneform bacteria and the usefulness of this character for classification of bacteria. The effects of culture conditions on

cellular fatty acid composition were investigated in order to determine the reliability of this characteristic for taxonomic purposes among coryneform bacteria.

MATERIALS AND METHODS

Abbreviations. The following culture collection abbreviations are used below: AJ, Central Research Laboratories, Ajinomoto Co., Inc., Kawasaki, Japan; ATCC, American Type Culture Collection, Rockville, Md.; CCEB, Culture Collection of Entomogenous Bacteria, Department of Insect Pathology, Institute of Entomology, Prague, Czechoslovakia; CCM, Czechoslovak Collection of Microorganisms, J. E. Purkyne University, Brno, Czechoslovakia; IAM, Institute of Applied Microbiology, University of Tokyo, Tokyo, Japan; IFM, Institute of Food Microbiology, Chiba University, Chiba, Japan; IFO, Institute for Fermentation, Osaka, Japan; LMD, Laboratorium voor Microbiologie der Landbouwhogeschool, Wageningen, The Netherlands; NCIB, National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, Scotland; NCPPB, National Collection of Plant Pathogenic Bacteria, Harpenden, England; NRRL, Northern Utilization Research and Development Division, U.S. Department of Agriculture, Peoria, Ill.

The following abbreviations are used for fatty acids: n, normal acid; i, iso acid; a, anteiso acid; 10-Me, 10-methyl acid; ch, ω -cyclohexyl fatty acid; 2-OH and h, 2-hydroxy fatty acid. For example, i-16, a-15, 10-Me-19, ch-17, and 18:1 are abbreviations for 14-methyl pentadecanoic acid, 12-methyl tetradecanoic acid, 10-methyl octadecanoic acid, ω -cyclohexyl undecanoic acid, and *n*-octadecenoic acid, respectively. Unsatu-

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rated fatty acids are straight-chain acids unless specified otherwise.

DNA, Deoxyribonucleic acid.

Bacterial strains. A total of 76 strains of coryneform bacteria belonging to the genera *Arthrobacter* (15 strains), *Brevibacterium* (12 strains), *Caseobacter* (1 strain), *Cellulomonas* (2 strains), *Corynebacterium* (27 strains), and *Curtobacterium* (19 strains) were used in this study. In the list below, the strains are arranged in groups according to the chemotaxonomic concepts proposed by Yamada and Komagata (49) and Keddle (21). (Names which do not appear on the Approved Lists of Bacterial Names [37] are enclosed in quotation marks.)

(i) **Group 1: *Corynebacterium*.** Group 1 contained "*Arthrobacter albidus*" CNF 092 (= NCIB 10266 = ATCC 15243) (23, 36), "*Brevibacterium alkanolyticum*" CNF 157 (= IFO 12323 = ATCC 21033) (36), *Brevibacterium ammoniagenes* CNF 096^T (type strain) (= AJ 1443^T = ATCC 6871^T = NCIB 8143^T) (23, 49), *Brevibacterium ammoniagenes* CNF 012 (= AJ 1444 = ATCC 6872) (36, 49), *Brevibacterium divaricatum* CNF 013^T (= AJ 1498^T = NRRL B-2312^T = ATCC 14020^T) (23, 36, 49), "*Brevibacterium flavum*" CNF 014 (= AJ 1510 = ATCC 14067) (23, 36, 49), "*Brevibacterium thioenitalis*" CNF 160 (= IFO 12115 = ATCC 19240) (30), *Corynebacterium diphtheriae* CNF 017 (= AJ 1414 = ATCC 11913) (49), *Corynebacterium equi* CNF 002^T (= AJ 1402^T = ATCC 6939^T = IAM 12426^T) (36, 49), *Corynebacterium equi* CNF 003 (= AJ 1403 = ATCC 10146 = IAM 12444) (49), *Corynebacterium equi* CNF 004 (= AJ 1372 = IAM 12445) (49), *Corynebacterium equi* CNF 005 (= AJ 1376 = IAM 12446) (49), *Corynebacterium equi* CNF 001 (= AJ 1378 = IAM 12443) (49), *Corynebacterium fascians* CNF 006^T (= AJ 1398^T = ATCC 12974^T = IAM 12427^T) (36, 49), *Corynebacterium flavesceus* CNF 011^T (= AJ 1415^T = ATCC 10340^T = IAM 1642^T) (36, 49), *Corynebacterium glutamicum* CNF 016^T (= AJ 1502^T = ATCC 13032^T = IAM 12435^T) (49), *Corynebacterium hoagii* CNF 007^T (= AJ 1408^T = ATCC 7005^T = IAM 12428^T) (49), "*Corynebacterium hoffmannii*" CNF 128 (= R. M. Keddle R76) (23), *Corynebacterium lilium* CNF 015^T (= AJ 1517^T = NRRL B-2243^T = ATCC 15990^T) (49), *Corynebacterium renale* CNF 009 (= AJ 1411 = ATCC 10848 = IAM 12430) (49), *Corynebacterium vitrum* CNF 008^T (= AJ 1525^T = ATCC 10234^T = IAM 12429^T) (36, 49), *Corynebacterium xerosis* CNF 010 (= AJ 1396 = ATCC 7711 = IAM 12431) (36, 49), *Corynebacterium xerosis* CNF 093 (= NCIB 9956 = CCM 1729 = IAM 12583), and *Corynebacterium xerosis* CNF 094 (= NCIB 10087 = NCTC 9755 = IAM 12584).

(ii) **Group 2: *Brevibacterium*.** Group 2 included *Brevibacterium linens* CNF 019^T (= AJ 1521^T = ATCC 9172^T = IAM 12437^T) (36, 49), and *Brevibacterium linens* CNF 018 (= AJ 1520 = ATCC 8377 = IAM 12436) (36, 49).

(iii) **Group 3: *Arthrobacter*.** Group 3 contained *Arthrobacter atrocyaneus* CNF 064^T (= IAM 12330^T = CCM 1645^T = ATCC 13752^T) (36), *Arthrobacter aureus* CNF 103^T (= IAM 12340^T = CCM 1649^T = ATCC 13344^T) (36, 49), *Arthrobacter citreus* CNF 021^T (= AJ 1423^T = ATCC 11624^T = IAM 12341^T) (36, 49), *Arthrobacter globiformis* CNF 022^T (= AJ 1422 = ATCC 8010^T = IAM 12438^T) (36, 49), *Arthrobacter nicotianae* CNF 104^T (= IAM 12342^T = CCM 1648^T =

ATCC 15236^T) (36, 49), *Arthrobacter ramosus* CNF 102^T (= IAM 12344^T = CCM 1646^T = ATCC 13727^T) (36, 49), *Arthrobacter tumescens* CNF 065 (= IAM 1447 = AJ 1549), (15, 49), *Arthrobacter tumescens* CNF 066 (= IAM 1458 = AJ 1460) (15, 49), *Arthrobacter ureafaciens* CNF 023^T (= AJ 1421^T = ATCC 7562^T = IAM 1658^T) (36, 49), *Arthrobacter* sp. strain CNF 108 (= AJ 1488 = ATCC 19098 = "*Brevibacterium sulfureum*" 8-3) (15, 36, 49), and "*Brevibacterium helvolum*" CNF 020 (= AJ 1445 = ATCC 11822 = IAM 1637) (35, 49).

(iv) **Group 4: *Cellulomonas*.** Group 4 included *Cellulomonas biazotea* CNF 024^T (= AJ 1569^T = ATCC 486^T = IAM 12106^T) (12, 36, 49) and *Cellulomonas fimi* CNF 025^T (= AJ 1571^T = ATCC 484^T = IAM 12107^T) (12, 36, 49).

(v) **Group 5: *Curtobacterium*.** Group 5 contained *Arthrobacter terregens* CNF 111^T (= NCIB 8909^T = ATCC 13345^T) (36), "*Corynebacterium barkeri*" CNF 095 (= NCIB 9658 = ATCC 15954 = IAM 12585) (36), *Curtobacterium albidum* CNF 028^T (= AJ 1472^T = IAM 1631^T = ATCC 15831^T) (24, 36, 49), *Curtobacterium citreum* CNF 026^T (= AJ 1469^T = IAM 1614^T = ATCC 15828^T) (24, 36, 49), *Curtobacterium citreum* CNF 061 (= AJ 1471 = IAM 12545) (24, 36, 49), *Corynebacterium flaccumfaciens* CNF 029 (= AJ 1400 = ATCC 6887) (36, 49), "*Curtobacterium insectiphilum*" CNF 059 (= AJ 1477 = IFM AM-23 = IAM 12546) (49), *Corynebacterium poinsettiae* CNF 027 (= AJ 1999 = CCM 1587 = ATCC 9682) (36, 49), *Curtobacterium pusillum* CNF 106^T (= IAM 1479^T = ATCC 19096^T) (15, 36, 43, 49), *Curtobacterium pusillum* CNF 107 (= IAM 1489 = ATCC 19097) (15, 36, 43, 49), *Curtobacterium saperdae* CNF 058^T (= AJ 3126^T = CCEB 366^T = ATCC 19272^T) (36), *Curtobacterium testaceum* CNF 032^T (= AJ 1466^T = IAM 1561^T = ATCC 15829^T) (24) (Yamada and Komagata [49] mis-cited *Curtobacterium testaceum* AJ 1464 [= Rp-1] as the type strain; in the original paper Komagata and Iizuka [24] designated strain Rp-3 [= AJ 1466 = IAM 1561] as the type strain of this species), *Curtobacterium testaceum* CNF 030 (= AJ 1464 = IAM 1537) (24, 49), *Curtobacterium testaceum* CNF 031 (= AJ 1465 = IAM 1549) (24), *Curtobacterium testaceum* CNF 033 (= AJ 1467 = IAM 1572) (24), *Curtobacterium testaceum* CNF 034 (= AJ 1468 = IAM 1583) (24), *Curtobacterium* sp. strain CNF 116 (= Nakayama et al. 114-2), *Curtobacterium* sp. strain CNF 162 (= IAM 1478 [*Brevibacterium helvolum* strain 129] = NCIB 10353) (15, 49), *Curtobacterium* sp. strain CNF 163 (= IAM 1434 [*Brevibacterium helvolum* strain 9-6] = ATCC 21342) (15, 49), *Curtobacterium* sp. strain CNF 164 (= IAM 1391 [*Brevibacterium helvolum* strain 123]) (15, 49), and *Curtobacterium* sp. strain CNF 165 (= IAM 1498 [*Brevibacterium helvolum* strain 9-8] = NCIB 10352) (15, 49).

(vi) **Group 6: coryneform bacteria with LL-diaminopimelic acid in their cell walls.** Group 6 included *Arthrobacter simplex* CNF 035^T (= AJ 1420^T = ATCC 6946^T = IAM 1660^T) (23, 36, 49), *Arthrobacter simplex* CNF 091 (= NCIB 9770 = IAM 12581) (23, 36), *Arthrobacter tumescens* CNF 067^T (= IAM 12345^T = CCM 1655^T = ATCC 6947^T) (23, 36, 49), "*Brevibacterium lipolyticum*" CNF 036 (= AJ 1450 = IAM 1398) (49), and "*Brevibacterium lipolyticum*" CNF 037 (= AJ 1451 = IAM 1413) (49).

(vii) **Group 7: coryneform bacteria with diaminobu-**

TABLE 1. Cellular fatty acid compositions of coryneform bacteria: type I (normal-unsaturated type)

Strain	Fatty acid composition (%)												
	Normal					Monounsaturated					10-methyl		
	n-14	n-15	n-16	n-17	n-18	15:1	16:1	17:1	18:1	19:1	10-Me-17	10-Me-18	10-Me-19
Subtype A													
<i>Corynebacterium glutamicum</i> CNF 016 ^T			41		1		1		56				
<i>Brevibacterium divaricatum</i> CNF 013 ^T			46						54				
" <i>Brevibacterium flavum</i> " CNF 014			46						54				
<i>Corynebacterium lilium</i> CNF 015 ^T			42						58				
<i>Corynebacterium renale</i> CNF 009			42				3		55				
<i>Corynebacterium xerosis</i> CNF 093	1		49						50				
<i>Corynebacterium xerosis</i> CNF 094	2		45				1		52				
" <i>Arthrobacter albidus</i> " CNF 092	2		46						52				
<i>Corynebacterium flavesces</i> CNF 011 ^T	2		24						74				
Subtype B													
<i>Caseobacter polymorphus</i> CNF 057 ^T	2		41						38				19
<i>Brevibacterium ammoniagenes</i> CNF 096 ^T			35		7		1		37				19
<i>Brevibacterium ammoniagenes</i> CNF 012	1		33		4		1		52				7
" <i>Brevibacterium thio</i> genitalis" CNF 160	1		30		2		1		57				7
Subtype C													
<i>Corynebacterium diphtheriae</i> CNF 017	3		45				48		3				
Subtype D													
<i>Corynebacterium xerosis</i> CNF 010			12	3	20				65				
" <i>Corynebacterium hofmannii</i> " CNF 128			32		10		13		45				
<i>Corynebacterium vitarumen</i> CNF 008 ^T			35		10		3		52				
Subtype E													
<i>Corynebacterium equi</i> CNF 002 ^T	5	1	32	2	2		13	1	16		3	1	23
<i>Corynebacterium equi</i> CNF 003	5	1	32	2	1		12	2	11		5	2	29
<i>Corynebacterium equi</i> CNF 004	7	2	34	1	1		11	1	9		7	2	24
<i>Corynebacterium equi</i> CNF 005	15	1	28		1		15		12		6		22
<i>Corynebacterium equi</i> CNF 001	6	4	33	3	1	1	22	4	11		4	2	9
" <i>Brevibacterium alkanolyticum</i> " CNF 158	8	3	30	2	1		15	2	8	1	7	2	20
Subtype F													
<i>Corynebacterium fascians</i> CNF 006 ^T	7	2	33	2	1		13	3	37	1			
<i>Corynebacterium hoagii</i> CNF 007 ^T	6	3	33	3	2		13	4	34	1			

tyric acid in their cell walls. Group 7 contained "*Corynebacterium aquaticum*" CNF 038 (= AJ 1413 = ATCC 14665 = IAM 12441) (23, 36, 49), *Corynebacterium insidiosum* CNF 101 (= AJ 1409 = ATCC 10253 = NCPPB 852) (36, 49), *Corynebacterium michiganense* CNF 039 (= AJ 1390 = ATCC 492) (36, 49), *Corynebacterium michiganense* CNF 097 (= AJ 1391 = ATCC 4450) (36, 49), *Corynebacterium michiganense* CNF 098 (= AJ 1392 = ATCC 7429) (36, 49), *Coryne-*

bacterium michiganense CNF 099 (= AJ 1393 = ATCC 7430 = NCPPB 861) (49), *Corynebacterium michiganense* CNF 100 (= AJ 1394 = ATCC 10202) (36, 49), *Corynebacterium* sp. strain CNF 133 (= R. M. Keddie A4) (23), and *Corynebacterium* sp. strain CNF 134 (= R. M. Keddie A112 [*Flavobacterium dehydrogenans*]) (23).

(viii) **Genus *Caseobacter*.** One member of the genus *Caseobacter* was studied, *Caseobacter polymorphus*

FIG. 1. Gas-liquid chromatograms of representatives of the six subtypes of cellular fatty acid type I (normal-unsaturated type). (A) Subtype A (*Corynebacterium glutamicum* CNF 016^T). (B) Subtype B (*Brevibacterium ammoniagenes* CNF 096^T). (C) Subtype C (*Corynebacterium diphtheriae* CNF 017). (D) Subtype D (*Corynebacterium xerosis* CNF 010). (E) Subtype E (*Corynebacterium equi* CNF 002^T). (F) Subtype F (*Corynebacterium fascians* CNF 006^T). A 2-m OV-1 column was used. For abbreviations of fatty acids, see text.

CNF 057^T (= LMD AC256^T = ATCC 33010^T = IAM 12549^T) (10).

(xi) **Unclassified strain.** We included one unclassified strain in this study, *Brevibacterium imperiale* CNF 105^T (= IAM 1654^T = ATCC 8365^T) (36, 49).

Culture media and cultivation of bacteria. Medium F was used throughout this study unless otherwise stated. Medium F contains 1% Bacto-Peptone (Difco

Laboratories, Detroit, Mich.), 0.5% yeast extract, 0.5% Casamino Acids (Difco), 0.5% malt extract, and 0.05% KH₂PO₄ (pH 7.2). Bacteria were grown in 500-ml conical flasks containing 200 ml of medium at 30°C for 24 h on a rotary shaker unless otherwise specified. *Corynebacterium diphtheriae* CNF 017 was grown at 37°C.

Preparation of cellular fatty acid methyl esters. Cellu-

TABLE 2. Cellular fatty acid compositions of coryneform bacteria: type II (iso-anteiso type)

Strain	Fatty acid composition (%)										
	Anteiso		Iso				Normal				
	a-15	a-17	i-14	i-15	i-16	i-17	n-14	n-15	n-16	n-17	n-18
Group 2: <i>Brevibacterium</i>											
<i>Brevibacterium linens</i> CNF 019 ^T	48	42	11	4	3	3					
<i>Brevibacterium linens</i> CNF 018	55	32	1	7	2	2					
Group 3: <i>Arthrobacter</i>											
<i>Arthrobacter globiformis</i> CNF 022 ^T	53	18	1	4	5	1	3	1	12		2
<i>Arthrobacter citreus</i> CNF 021 ^T	57	8	2	7	7	1	2	1	11		
<i>Arthrobacter nicotianae</i> CNF 104 ^T	54	11	2	6	10	1	3		12		
<i>Arthrobacter tumescens</i> CNF 065	44	16		17	4		1	1	8	5	1
<i>Arthrobacter tumescens</i> CNF 066	39	17		13	4	4	1		11		5
<i>Arthrobacter atrocyaneus</i> CNF 064 ^T	36	31	1	13	7	6			2		1
" <i>Brevibacterium helvolum</i> " CNF 020	53	18	1	17	3	3	1		4		
<i>Arthrobacter ureafaciens</i> CNF 023 ^T	58	28	1	5	6	2			1		1
<i>Arthrobacter ramosus</i> CNF 102 ^T	69	26	1	2					1		
<i>Arthrobacter aurescens</i> CNF 103 ^T	66	26		2	4	1			1		
<i>Arthrobacter</i> sp. strain CNF 108	69	10	1	6	5	1	2		6		
Group 4: <i>Cellulomonas</i>											
<i>Cellulomonas fimi</i> CNF 025 ^T	46	12	2	8	4	1	5	2	10	4	6
<i>Cellulomonas biazotea</i> CNF 024 ^T	48	13	3	5	4	1	3	5	8	5	4
Group 5: <i>Curtobacterium</i>											
<i>Curtobacterium citreum</i> CNF 026 ^T	36	36	1	2	11	1	1	1	8	1	2
<i>Curtobacterium citreum</i> CNF 061	40	39	1	4	10	1		1	3	1	2
" <i>Curtobacterium poinsettiae</i> " CNF 027	32	45		2	13	1			3		2
<i>Curtobacterium albidum</i> CNF 028 ^T	33	46	1	2	10	3		1	3		2
" <i>Curtobacterium flaccumfaciens</i> " CNF 029	52	33	1	3	5	1			2		2
" <i>Curtobacterium insectiphilium</i> " CNF 059	42	34		1	14	2			4		3
" <i>Corynebacterium barkeri</i> " CNF 095	34	46	1		16				3		
<i>Curtobacterium testaceum</i> CNF 032 ^T	28	37		4	19	2		2	3	1	2
<i>Curtobacterium testaceum</i> CNF 030	23	46		2	16	4			5		2
<i>Curtobacterium testaceum</i> CNF 031	28	44	1	1	17	2	1	1	4	1	1
<i>Curtobacterium testaceum</i> CNF 033	28	37		4	19	2		2	3	1	2
<i>Curtobacterium testaceum</i> CNF 034	24	48		1	16	2		1	4	1	3
<i>Arthrobacter terregens</i> CNF 111 ^T	26	55		1	15				1	1	
<i>Curtobacterium saperdae</i> CNF 058 ^T	43	26		2	16	2			7		2
<i>Curtobacterium</i> sp. strain CNF 116	51	20		3	13	8			1	1	2
<i>Curtobacterium</i> sp. strain CNF 162	43	35			19				2		
<i>Curtobacterium</i> sp. strain CNF 163	40	38		1	17	1			3		
<i>Curtobacterium</i> sp. strain CNF 164	43	37		1	14	1			2		
Group 7: coryneform bacteria with diaminobutyric acid											
<i>Corynebacterium michiganense</i> CNF 039	56	33			9				2		
<i>Corynebacterium michiganense</i> CNF 097	52	34			9			1	2		
<i>Corynebacterium michiganense</i> CNF 098	48	36			8			3	3		
<i>Corynebacterium michiganense</i> CNF 099	71	17			7				3		
<i>Corynebacterium michiganense</i> CNF 100	40	35			14				5		
" <i>Corynebacterium aquaticum</i> " CNF 038	29	54		1	14	1			1		
<i>Corynebacterium insidiosum</i> CNF 101	47	45			4				1		
<i>Corynebacterium</i> sp. strain CNF 133	59	24		2	8				1		
<i>Corynebacterium</i> sp. strain CNF 134	38	33		6	19				1		
Unclassified											
<i>Brevibacterium imperiale</i> CNF 105	29	55			10				3		

lar fatty acid methyl esters were prepared as follows. Cells were harvested by centrifugation, washed twice with distilled water, and lyophilized. Fatty acid methyl esters were liberated from 20 mg of lyophilized cells by methanolysis at 100°C for 3 h with 2.5 ml of 5% anhydrous methanolic HCl and were extracted three times with petroleum ether (3 ml each time).

Analytical methods. Fatty acid methyl esters were determined with a Shimadzu model GC-6A gas chromatograph and a Shimadzu model CR1-A chromatograph-data processor. Two kinds of glass columns were used to separate the fatty acid methyl esters. One column (5 m by 3 mm) was packed with 10% diethylenglycol succinate on Chromosorb W (type AW-

FIG. 2. Gas-liquid chromatograms of representatives of cellular fatty acid type II (iso-anteiso type). (A) *Brevibacterium linens* CNF 019^T. (B) *Arthrobacter globiformis* CNF 022^T. (C) *Cellulomonas fimi* CNF 025^T. (D) *Curtobacterium citreum* CNF 026^T. A 5-m diethyleneglycol succinate column was used. For abbreviations of fatty acids, see text.

DMCS; 80 to 100 mesh), and the other column (2 m by 3 mm) was packed with 5% OV-1 on Chromosorb W (type AW-DMCS; 80 to 100 mesh). Comparative analyses with the two types of columns were useful for fatty acid identification. Fatty acid methyl esters, L-203, L-205, and BC MIX-L (obtained from Applied Sciences Laboratories, State College, Pa.) were used as standards.

Methyl esters of hydroxy fatty acids were separated from methyl esters of nonpolar acids by thin-layer chromatography on Kieselgel F-254 (E. Merck AG, Darmstadt, West Germany). The chromatograms were developed with a petroleum ether-diethyl ether (1:1, vol/vol) solvent system. Fractions were scraped from the chromatograms, stained with rhodamine 6G, and compared with the reference hydroxy fatty acid fractions.

Gas-liquid chromatography-mass spectrometry was performed with a model GCMS-9000S spectrometer (Shimadzu-LKB) and a PAC 90 computer system, as described previously (43). Some fatty acid methyl esters were analyzed with a Hitachi model 163 gas chromatograph and a JEOL model JMS-D300 gas

chromatograph-mass spectrometer. Both instruments were equipped with a capillary column (50 m by 0.25 mm) of Carbowax 20M.

Effects of culture conditions. The effects of culture conditions on cellular fatty acid composition were determined for some strains. Growth temperature (20, 27, 30, and 37°C), culture age (6, 12, 24, 48, and 120 h), and growth media (medium F and medium G) were varied independently. Medium G contained 1% glucose, 0.5% Proteose Peptone (Difco), 0.5% sodium glutamate, 0.1% K₂HPO₄, 0.02% MgSO₄ · 7H₂O, and 0.01% KCl; the pH was adjusted to 7.2 (49).

RESULTS

We identified four major groups of coryneform bacteria based on cellular fatty acid constituents. Each of these is described below.

Type I. Type I was characterized by the presence of saturated and monounsaturated straight-chain C₁₆ and C₁₈ fatty acids. In addition, some strains contained 10-methyl fatty

FIG. 3. Gas-liquid chromatogram of cellular fatty acids extracted from *Curtobacterium pusillum* CNF 106^T (cellular fatty acid type III). A 5-m diethyleneglycol succinate column was used. For abbreviations of fatty acids, see text.

acids. Major components were *n*-hexadecanoic acid (*n*-16 acid), *n*-octadecenoic acid (18:1 acid), *n*-octadecanoic acid (*n*-18 acid), *n*-hexadecanoic acid (16:1 acid), and 10-methyl octadecanoic acid (10-Me-19 acid). The type I cellular fatty acid composition was found in all group 1 strains (49) and the strain of *Caseobacter* tested (Table 1). Type I strains were further divided into six subtypes (subtypes A through F) on the basis of the relative quantities of the acids that were found. Six representative gas-liquid chromatograms of these subtypes are shown in Fig. 1. The strains of subtype A contained exclusively *n*-16 and 18:1 acids. Nine strains, including *Corynebacterium glutamicum* CNF 016^T, were subtype A strains. Subtype B differed from subtype A by the occurrence of 10-Me-19 acid and included four strains. There was only one strain of subtype C, in which the fatty acids were mostly 16:1 and *n*-16 acids. The three subtype D strains had 18:1, *n*-16, and *n*-18 acids and differed from subtype A strains by the presence of *n*-18 acid. In subtypes E and F, odd-numbered straight-chain acids, both saturated and unsaturated, were found, although the amounts were small. Subtype E strains contained moderate amounts of 10-methyl fatty acids, whereas none of these fatty acids were found in subtype F strains.

Type II. Type II cellular fatty acid composi-

tion included mainly anteiso and iso acids and was found in all tested strains belonging to group 2 (*Brevibacterium*), group 3 (*Arthrobacter*), and group 4 (*Cellulomonas*), 18 of 21 group 5 (*Curtobacterium*) strains tested, and all group 7 (coryneform bacteria with diaminobutyric acid in their cell walls) strains tested (Table 2). Gas-liquid chromatograms of the fatty acids of four strains having type II fatty acid composition are shown in Fig. 2. The major components of this type were 12-methyl tetradecanoic acid (*a*-15 acid) and 14-methyl hexadecanoic acid (*a*-17 acid). The content of normal acids varied among strains. Normal acids in the two strains belonging to the genus *Cellulomonas* (group 4) accounted for approximately 25% of the total fatty acids detected, whereas two strains of *Brevibacterium linens* (group 2) possessed no normal acids. *Brevibacterium imperiale* CNF 105^T, which does not belong to any of the seven groups of Yamada and Komagata (49), also had type II fatty acid composition.

Type III. As previously reported (43), two strains of *Curtobacterium pusillum* possessed large amounts of ω -cyclohexyl undecanoic acid (*ch*-17 acid), which is the characteristic fatty acid of type III (Table 3). A gas-liquid chromatogram of the cellular fatty acids of *Curtobacterium pusillum* CNF 106^T is shown in Fig. 3.

TABLE 3. Cellular fatty acid compositions of coryneform bacteria: type III (ω -cyclohexyl type)

Strain	Fatty acid composition (%)									
	Cyclohexyl		Anteiso		Iso			Normal		
	<i>ch</i> -17	<i>ch</i> -19	<i>a</i> -15	<i>a</i> -17	<i>i</i> -15	<i>i</i> -16	<i>i</i> -17	<i>n</i> -15	<i>n</i> -16	<i>n</i> -17
<i>Curtobacterium pusillum</i> CNF 106 ^T	64	3	12	17	1	2	1		1	
<i>Curtobacterium pusillum</i> CNF 107	51	1	21	20	1	2		1	1	1
<i>Curtobacterium</i> sp. strain CNF 165	5		28	41	3	11	2		7

FIG. 4. Gas-liquid chromatogram of cellular fatty acids extracted from *Arthrobacter simplex* CNF 035^T (cellular fatty acid type IV). A 5-m diethyleneglycol succinate column was used. For abbreviations of fatty acids, see text.

Curtobacterium sp. strain CNF 165 also contained ch-17 acid, but it accounted for only 5% of the total cellular fatty acids. ω -Cyclohexyl fatty acids were not found in the other coryneform bacteria examined.

Type IV. The type IV fatty acid pattern was complex (Fig. 4). Many peaks corresponding to iso, anteiso, normal, saturated, unsaturated, 10-methyl, and 2-hydroxy fatty acids were found in all group 6 strains tested. Group 6 strains are the strains with LL-diaminopimelic acid in their cell walls (23, 49). Table 4 shows the fatty acid compositions of the strains with type IV cellular fatty acids.

Effects of culture conditions. Selected strains with type I and II cellular fatty acid compositions were used for an analysis of cellular fatty acids under various growth conditions.

Representative strains of the six subtypes in type I were studied. In general, the composition of the culture medium did not affect the fatty acid composition. However, growth temperature did affect the cellular fatty acid composition. When cells were grown at higher temperatures, the content of saturated (normal and 10-methyl) acids increased, and the content of unsaturated acids decreased (Fig. 5). The contents of 10-methyl fatty acids in the subtype B and E strains tested increased as the cells aged. However, the content of 10-methyl acids plus unsaturated acids was rather constant through 120 h of cultivation (Fig. 6).

Among the strains with type II fatty acid composition, nine representative strains were examined. The fatty acid compositions of these nine strains did not change through 120 h of cultivation, but there was a marked effect of culture medium composition. This effect differed among the strains (Fig. 7).

In four test strains the ratio of a-17 acid to a-15

acid increased as the growth temperature increased (Table 5).

DISCUSSION

The four types of cellular fatty acid composition in coryneform bacteria appear to be distinct and do not overlap, even when the culture conditions are changed slightly. Most of the strains studied belonged to type I (normal-unsaturated type) or type II (iso-anteiso type). These two types are quite different and are discussed separately below.

Type I fatty acid composition was found in all coryneform bacteria that have *meso*-diaminopimelic acid and arabinogalactan in their cell walls (23, 36). Some strains of *Nocardia* and related taxa that have the same cell wall components also possess type I subtype E cellular fatty acid composition (unpublished data). The apparent correlation between these two characters suggests that the cellular fatty acid composition of these organisms is taxonomically significant. Further study of this relationship among *Rhodococcus* strains will be required (30).

Type I subtype A, which is found in most glutamic acid-producing bacteria, uniformly consists of n-16 and 18:1 acids but not n-18 acid; on the other hand, n-18 is found in subtype D.

Subtype C, which is found only in *Corynebacterium diphtheriae* CNF 017, contains small amounts of both saturated and unsaturated C₁₈ fatty acids, as observed by Barksdale et al. (1). This pattern is considered to be characteristic for *Corynebacterium diphtheriae*. Further studies will be required to reveal the relationship of subtypes A through D.

Among the six subtypes of type I, both subtype B and subtype E contain 10-methyl fatty acids. 10-Methyl fatty acids are formed by the

TABLE 4. Cellular fatty acid compositions of coryneform bacteria: type IV (complex type)

Strain	Fatty acid composition (%)													
	Anteiso		Iso								Normal			
	a-15	a-17	i-14	i-15	i-16	i-17	i-18	i-19	i-16:1	i-19:1	n-14	n-15	n-16	n-17
<i>Arthrobacter simplex</i> CNF 035 ^T	2 ^a	18	T	1	9	3	1	1		1	T	1	7	4
<i>Arthrobacter simplex</i> CNF 091	1	4	1	13	18	13	1				T	1	4	7
<i>Arthrobacter tumescens</i> CNF 067 ^T	11	7	3	39	7	2	T		4		1	1	2	4
" <i>Brevibacterium lipolyticum</i> " CNF 036	1	14		2	12	6	2	1		1		1	4	3
" <i>Brevibacterium lipid</i> " CNF 037	1	13		2	11	8	1	1		1		T	5	3

^a Values are percentages of the total fatty acids detected. T, Trace (less than 0.5%)

introduction of a methyl group from *S*-adenosyl-methionine to the double bonds of the corresponding unsaturated acids (19, 27). In the present study, the content of 10-methyl acids increased with culture age, but the amount of 10-methyl acids plus unsaturated acids was almost constant. Therefore, 10-methyl acids can be considered to be derivatives of the corresponding unsaturated acids. Because of this relationship, in quantitative comparisons of cellular fatty acid compositions the total contents of 10-methyl acids and unsaturated acids should be considered together.

The difference between subtype E and subtype F is solely the presence of 10-methyl acids. When the contents of 10-methyl acids are added to those of the corresponding unsaturated acids, subtype E shows a composition similar to that of subtype F. In fact, the strains of both subtypes fall into one cluster on the basis of DNA homology (42), and they are the only strains among the corynebacteria that possess the glycolyl type of cell wall (44, 45; unpublished data). Therefore, strains of subtype E and subtype F are more closely related to each other than to other subtypes.

The contents of unsaturated acids decreased in proportion to increases in growth temperature (Fig. 6). This phenomenon has also been observed in *Escherichia coli* (19). Kawaguchi et al. (20) found the same thermal regulation in a cell-free system of fatty acid biosynthesis with *Brevibacterium ammoniagenes* CNF 096^T.

The distribution of type II cellular fatty acids was in agreement with the results obtained by other investigators, who also found that strains of *Brevibacterium linens* (2, 6), *Arthrobacter* (2), *Cellulomonas* (29), *Curtobacterium* (4), and coryneform bacteria with the diaminobutyric acid type of peptidoglycan (5) contain iso-anteiso cellular fatty acids. Although the major components are anteiso acids (a-15 and a-17 acids), some genera have additional distinctive differences in composition. Strains of *Brevibacterium linens* did not possess normal acids, whereas relatively large amounts of normal acids were found in the two strains of *Cellulomonas*. In

strains of the genus *Curtobacterium* (group 5), i-16 acid was always the third-most-abundant acid after the anteiso acids.

On the basis of cellular fatty acid composition, five strains of *Curtobacterium testaceum*, *Arthrobacter terregens* CNF 111^T, *Curtobacterium saperdae* CNF 058^T, and *Curtobacterium* sp. strain CNF 116, which possess a glycolyl cell wall type (44, 45; unpublished data), were not distinguished from other *Curtobacterium* strains which have an acetyl cell wall type.

Strains of both *Brevibacterium linens* and the genus *Corynebacterium* commonly have meso-diaminopimelic acid as the principal amino acid of the peptidoglycan. The chemotaxonomic differences between these two genera include the presence of arabinogalactan in the cell wall (23, 36) and the occurrence of mycolic acids (23). Strains of *Brevibacterium linens* can be clearly differentiated from corynebacteria (group 1) and a strain of *Caseobacter* by cellular fatty acid composition. The former have type II fatty acid composition, and the latter have type I.

The composition of iso-anteiso fatty acids in bacteria is ascribed to the primer specificity of the starting enzyme for fatty acid biosynthesis. Even-numbered iso acids, odd-numbered iso acids, and odd-numbered anteiso acids are synthesized from the primers valine, leucine, and isoleucine, respectively (18, 27). Therefore, differences in medium composition may be expected to change the cellular fatty acid composition, as shown in Fig. 7.

Table 5 shows that coryneform bacteria with type II fatty acid composition synthesize proportionately more longer fatty acids as the growth temperature increases. This phenomenon was observed in some thermophilic bacteria by Oshima and Miyagawa (33). It is evident that taxonomic comparisons of fatty acid composition should only be done with data obtained from cells grown under the same cultural conditions.

Type III cellular fatty acid composition is characterized by the occurrence of ω -cyclohexyl fatty acids. These unusual fatty acids were found only in three *Curtobacterium* strains (Ta-

TABLE 4—Continued

Fatty acid composition (%)															
							10-Methyl				2-Hydroxy				
n-18	n-19	15:1	16:1	17:1	18:1	19:1	10-Me-17	10-Me-18	10-Me-19	10-Me-20	h-a-17	h-i-16	h-a-17	h-n-16	h-n-17
11	1		1	2	13	T	T	1	12	T	4	2	1	1	
3			2	5	10	T	T	3	6	T	T	1	1	T	
1		1	3	2	4	T		5	T					T	
4	T		1	5	28	1			T	2	4	3	1	1	T
9	T		1	2	15	T	T	1	12	1	4	3	2	1	

ble 3). The presence of ω -cyclohexyl fatty acids is considered to be a specific character of *Curtobacterium pusillum*. This species meets the concept of the genus *Curtobacterium* in phenotype and in chemotaxonomic profiles (49). *Curtobacterium* sp. strain CNF 165, which possesses a small amount of ch-17 acid, seems to be intermediate between type II and type III. When ω -cyclohexyl fatty acids are excluded, the fatty acid components of these three *Curtobacterium* strains are essentially iso-anteiso acids, as for type II. These similarities indicate that type III is a variant of type II.

ω -Cyclohexyl alkanes are found in crude oil, but until 1965 the possible origins of these compounds were unknown (28). Therefore, it is of interest that strains of *Curtobacterium* with ω -cyclohexyl fatty acids have been isolated from oil brine in Japan (15).

Type IV (complex type) is found only in the coryneform bacteria with LL-diaminopimelic acid-containing peptidoglycan (group 6). The cellular fatty acid components of *Arthrobacter simplex* CNF 035^T agreed well with the fatty acid components of phospholipids of the same strain studied by Yano et al. (51). *Arthrobacter simplex* CNF 035^T and "*Brevibacterium lipolyticum*" CNF 036 and CNF 037 formed a single cluster based on DNA homology (42). *Arthrobacter simplex* CNF 035^T, "*Brevibacterium lipolyticum*" CNF 036, and *Arthrobacter tumescens* CNF 067 share the same menaquinone [MK-8(H₄)] (3, 50). These results support the homogeneity of group 6.

Our data indicate that when culture conditions are standardized, the cellular fatty acid profile becomes a reliable characteristic for taxonomic comparisons among coryneform bacteria. The

FIG. 5. Effect of growth temperature on the cellular fatty acid compositions of six type I coryneform bacterial strains (normal-unsaturated type).

FIG. 6. Effect of culture age on the cellular fatty acid compositions of six type I coryneform bacterial strains (normal-unsaturated type).

TABLE 5. Effect of growth temperature on the fatty acid compositions of type II coryneform bacteria (iso-anteiso type)

Strain	Growth temp (°C)	Fatty acid composition (%)								Ratio of a-17 to a-15
		Anteiso		Iso				Normal		
		a-15	a-17	i-14	i-15	i-16	i-17	n-14	n-16	
<i>Brevibacterium linens</i> CNF 019 ^T	20	64	27		4	3				0.43
	27	56	32		6	4	2			0.58
	37	48	40		3	6	1			0.83
“ <i>Brevibacterium helvolum</i> ” CNF 020	20	71	9	2	9	3		2	3	0.13
	27	61	16	1	8	7	1	1	4	0.26
	37	58	15	1	15	6	3		2	0.27
<i>Arthrobacter ureafaciens</i> CNF 023 ^T	20	78	11	1	2	5			3	0.14
	27	63	19	1	5	9			1	0.30
	37	59	27	1	4	7	1		2	0.46
“ <i>Corynebacterium aquaticum</i> ” CNF 038	20	66	15	1	1	10		2	2	0.23
	27	33	36		5	20	3		1	1.08
	37	26	50		2	18	2		2	1.93

four major types of fatty acid composition correlate well with other chemotaxonomic data. Types I, II, and IV also agree completely with the dendrogram based on 16S ribosomal ribonucleic acid cataloging of Stackebrandt et al. (41). Therefore, we believe that cellular fatty acid analysis will be extremely useful in overcoming many of the difficulties that have been encoun-

tered in identifying and classifying the coryneform bacteria.

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