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Differentiation of Species Combined into the *Burkholderia cepacia* Complex and Related Taxa on the Basis of Their Fatty Acid Patterns

Eva Krejčí[1,2]* and Reiner M. Kroppenstedt[2]

Institute of Public Health, Centre for Microbiology, Parasitology and Immunology, Partyzánské nám. 7, 702 00 Ostrava, Czech Republic,[1] and DSMZ—Deutsche Sammlung von Mikroorganismen und Zellkulturen, Mascheroder Weg 1b, 38124 Braunschweig, Germany[2]

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Using the established commercial system Sherlock (MIDI, Inc.), cellular fatty acid methyl ester analysis for differentiation among *Burkholderia cepacia* complex species was proven. The identification key based on the diagnostic fatty acids is able to discern phenotypically related *Ralstonia pickettii* and *Pandoraea* spp. and further distinguish *Burkholderia pyrrocinia*, *Burkholderia ambifaria*, and *Burkholderia vietnamiensis*.

At present, nine *Burkholderia* species are combined into the *Burkholderia cepacia* complex (BCC): *B. cepacia*, *B. multivorans*, *B. cenocepacia*, *B. stabilis*, *B. vietnamiensis*, *B. dolosa*, *B. ambifaria*, *B. anthina*, and *B. pyrrocinia* (2, 12–14). In some cases, the severity of BCC infection is closely connected with one particular species. The proper identification of likely BCC isolates is crucial for patients suffering from cystic fibrosis (CF) (9).

Identification of BCC isolates to the species level based on the results obtained by genotypic methods, such as restriction fragment length polymorphism analysis of the *recA* gene or 16S rRNA genes (17, 18) and *recA* gene-based PCR (4), is mostly done with research tools provided in reference centers. Phenotypic testing is still frequently used in routine laboratories. Because of constantly changing taxonomy, such identification could be inaccurate. Databases of commonly used commercial kits are often incomplete and may even be insufficient to properly discern BCC isolates from other phylogenetically related isolates or from *Ralstonia pickettii* and *Pandoraea* spp. commonly isolated from clinical human sources.

Cellular fatty analysis using semiautomated gas chromatography by the microbial identification system (MIS) Sherlock (MIDI, Inc., Newark, Del.) is a relatively rapid and cost-effective method widely available and suitable for clinical laboratories. Identification based on the species-specific differences in fatty acid composition in cell lipidic structure was proved to be a good taxonomic marker (15). Contrary to phenotyping, previous studies have shown that analysis of cellular fatty acid components was able to distinguish between the genera *Burkholderia*, *Ralstonia*, and *Pandoraea* (1, 19). Fatty acids of the BCC are rather uniform; nevertheless, species-specific differences useful to separate *B. anthina* or *B. ambifaria* from *B. cepacia* and *B. cenocepacia* were observed (2, 13). So far there are no reports that include and compare the fatty acid compositions of all known BCC species and related taxa under standardized culture conditions. In this study, we tested whether comparing fatty acid profiles obtained on MIS Sherlock could yield the ability to sufficiently discriminate between BCC species and other closely related taxa.

Type strains (Tables 1 and 2) and a set including 47 well-characterized clinical isolates recovered during a half-year survey in 2002 in two town hospitals and several ambulatory medical practices in our region (Ostrava, Czech Republic) were studied. Isolates (one per patient) originated primarily from non-CF patients (sputum, blood cultures, wound and vaginal swabs, urine, catheter isolates, and isolates from the hospital environment; two *B. cenocepacia* strains were obtained from the sputum of CF patients). Tentative phenotypic identification as BCC was performed by commercially available biochemical tests (NEFERMtest24; Pliva-Lachema Diagnostika) and additional conventional testing (6; unpublished data). BCC species were determined by *recA* gene-based PCR assay as described previously (4). Differences in fatty acid compositions of other organisms (*Inquilinus limosus* and members of the genera *Cupriavidus*, *Achromobacter*, and *Delftia*) which were previously found in the respiratory tracts of cystic fibrosis patients or misidentified as BCC or *Pandoraea* spp. (1, 12) were also compared (Tables 1 and 2).

Because the fatty acid composition in the bacterial cell is influenced to a great extent by different culture conditions, the manufacturer's culture conditions given for each MIS library must always be followed to get usable identification results. To prepare whole-cell fatty acid methyl esters (FAME), one loop of fresh growing cultures of studied strains was harvested. Fatty acids were then saponified by means of heated alkaline hydrolysis, converted to the methyl esters, and extracted following the instructions of the MIS Sherlock operating manual (MIS operating manual, version 4.0; MIDI, Inc. Newark, Del.; partly available online). Samples were analyzed on a 25-m by 0.2-mm phenylmethyl siloxane capillary column (HP Ultra 2) using an HP 6890 gas chromatograph equipped with a flame-ionization detector. Gained FAME profiles could generally be compared in two standard libraries, CLIN50 and TSBA50. According to the quadrant plate-streaking technique, where four quadrants with different densities of cells are created, the third quadrant, which usually contains cells in the desired late

* Corresponding author. Mailing address: Institute of Public Health, Centre for Microbiology, Parasitology and Immunology, Partyzánské nám. 7, 702 00 Ostrava, Czech Republic. Phone: 420 596 200 425. Fax: 420 596 118 661. E-mail: eva.krejci@zuova.cz.

TABLE 1. Fatty acid compositions of all nine BCC type strains[a]

Fatty acid[b] or comparison	Fatty acid composition (%)[c] or species identification of:											
	B. cepacia	*B. multivorans*		*B. cenocepacia*		*B. stabilis*		*B. vietnamiensis*	*B. dolosa*	*B. ambifaria*	*B. anthina*	*B. pyrrocinia*
	DSM 7288T	DSM 13243T	17 isolates	CCM 4899T	15 isolates	CCM 4900T	15 isolates	DSM 11319T	LMG 18943T	CCUG 44356T	CCUG 46047T	DSM 10685T
Saturated												
12:0	tr[d] (1)	ND[e]	ND	ND	tr (2 CF[i])	ND	ND	0.1 ± 0.0 (2)	tr (2)	**0.9 ± 0.1**	ND	**2.8 ± 0.2**
14:0	3.8 ± 0.1	5.2 ± 0.1	3.6 ± 0.2	3.4 ± 0.2	3.7 ± 0.2	4.1 ± 0.1	3.9 ± 0.2	4.0 ± 0.2	3.9 ± 0.2	**2.9 ± 0.2**	4.1 ± 0.2	**0.5 ± 0.0**
16:0	27.2 ± 2.1	30.2 ± 1.3	22.9 ± 1.1	23.4 ± 2.4	19.0 ± 1.0	19.7 ± 0.7	20.5 ± 0.9	21.2 ± 0.2	26.0 ± 6.6	17.4 ± 0.6	20.5 ± 1.2	18.1 ± 0.2
17:0	0.3 ± 0.0 (2)	ND	0.2 (1)	ND	0.2 ± 0.0 (3)	*0.3 ± 0.1*	*0.2 ± 0.0 (6)*	0.4 ± 0.1	ND	0.1 (1)	0.2 ± 0.0 (2)	0.4 ± 0.1
18:0	1.5 ± 0.1	0.1 ± 0.1 (2)	1.4 ± 0.2	0.9 ± 0.2	0.9 ± 0.2	0.8 ± 0.1	0.9 ± 0.1	**3.6 ± 0.5**	0.6 ± 0.2	0.6 ± 0.1	0.6 ± 0.1	0.7 ± 0.0
Unsaturated												
13:1 AT 12-13	1.2 ± 1.0	1.0 ± 0.8	0.8 ± 0.7 (6)	1.6 (1)	0.7 ± 0.6 (9)	1.0 ± 0.8	0.8 ± 0.8 (5)	1.1 ± 0.9	1.0 ± 0.8	1.2 ± 1.0	1.2 ± 1.0	0.6 ± 0.6
16:1ω5c	0.2 (1)	0.7 ± 0.0	0.2 (1)	ND	0.2 ± 0.0 (5)	0.2 ± 0.1 (2)	0.3 ± 0.3 (4)	ND	0.4 ± 0.2	0.3 ± 0.1	0.4 ± 0.1 (2)	0.3 ± 0.1
16:1ω7c[f]	18.7 ± 5.2	27.6 ± 2.0	17.4 ± 1.4	12.6 ± 1.3	16.3 ± 2.9	18.6 ± 2.9	18.5 ± 2.5	23.2 ± 1.6	17.8 ± 1.7	13.5 ± 4.1	17.9 ± 6.7	19.1 ± 2.9
18:1ω7c	26.9 ± 4.4	16.2 ± 1.1	40.8 ± 2.1	29.3 ± 2.3	37.4 ± 3.8	35.1 ± 1.1	33.0 ± 4.1	31.8 ± 0.6	27.9 ± 6.7	36.0 ± 1.6	32.6 ± 1.7	37.3 ± 1.2
Hydroxy												
10:0 3OH	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
12:0 2OH	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
14:0 2OH	ND	0.1 ± 0.0 (2)	0.1 (1)	*0.5 ± 0.1*	*0.3 ± 0.1 (9)*	ND	ND	ND	0.2 ± 0.1 (2)	ND	ND	ND
14:0 3OH[g]	5.1 ± 0.1	5.8 ± 0.3	4.7 ± 0.4	5.0 ± 0.1	5.0 ± 0.4	5.5 ± 0.2	4.8 ± 0.4	5.0 ± 0.2	5.4 ± 0.0	4.8 ± 0.4	5.7 ± 0.4	4.9 ± 0.5
16:0 2OH	0.4 ± 0.1	0.3 ± 0.0	0.5 ± 0.2 (12)	0.6 ± 0.0	1.0 ± 0.3	1.0 ± 0.2	1.2 ± 0.2	0.7 ± 0.0	0.8 ± 0.4	0.8 ± 0.1	0.8 ± 0.2	1.4 ± 0.1
16:0 3OH	3.2 ± 0.6	3.3 ± 0.7	3.8 ± 0.5	3.9 ± 0.8	3.8 ± 0.4	4.3 ± 1.7	3.9 ± 0.3	3.6 ± 0.8	3.4 ± 0.8	4.0 ± 0.7	3.5 ± 1.1	3.4 ± 0.7
16:1 2OH	0.5 ± 0.0	1.7 ± 0.2	0.5 ± 0.2 (11)	0.5 ± 0.1	1.1 ± 0.3	1.0 ± 0.1	1.6 ± 0.4	0.8 ± 0.1	1.2 ± 0.4	0.8 ± 0.1	0.7 ± 0.1	1.7 ± 0.2
18:1 2OH	0.9 ± 0.3	*0.6 ± 0.1*	*0.6 ± 0.3 (11)*	1.3 ± 0.2	2.3 ± 0.7	2.0 ± 0.2	2.2 ± 0.7	1.2 ± 0.1	1.5 ± 1.0	2.1 ± 0.1	2.2 ± 0.5	2.2 ± 0.1
Cyclo												
17:0 cyclo	6.7 ± 4.2	5.1 ± 2.1	2.1 ± 1.2	11.1 ± 0.6	5.3 ± 3.1	3.9 ± 2.6	6.5 ± 3.2	2.6 ± 1.2	6.1 ± 2.0	10.2 ± 1.7	6.9 ± 4.3	4.9 ± 1.9
19:0 cyclo ω8c	3.4 ± 2.4	1.3 ± 0.3	1.7 ± 0.9	6.6 ± 0.8	3.3 ± 2.2	2.1 ± 1.5	2.6 ± 1.3	0.7 ± 0.4	3.4 ± 1.3	5.1 ± 2.4	2.9 ± 2.4	1.2 ± 0.7
Library comparison												
TSBA50[h]	*B. cepacia*/ *B. gladioli*	*Serratia odorifera*/ *Raoultella terrigena*	*B. cepacia*/ *B. caryophylli*	*B. cepacia*	*B. cepacia*	*B. cepacia*	*B. cepacia*	*B. cepacia*	*B. cepacia*/ *B. gladioli*	*B. cepacia*	*B. cepacia*	*B. pyrrocinia*/ *B. cepacia*

[a] Fatty acid compositions of species related to the BCC are given in Table 2. Fatty acids present in all species in an amount lower than 0.5% were omitted. Cultivation conditions for the TSBA50 standard library were used.

[b] In a designation, the first value is number of C atoms in the fatty acid. The number following the colon is the number of double-bound C atoms. The value following the letter ω signifies the position in the carbon chain (counted from opposite the carboxyl group). The number preceding OH indicates the number of OH groups. cyclo, cyclopropane circle; c, *cis* conformation.

[c] Values are means ± standard deviations. Values in parentheses show how many times the fatty acid appeared. Mean values were calculated either for three independent analyses (type strains) or for several isolates (one isolate each for *B. multivorans, B. cenocepacia*, and *B. stabilis*) by using FAME data from the standard MIS Sherlock composition report. With the aid of MS Excel software, the standard deviations were calculated according to the formula [sgrt $(\Sigma(x - x')^2/(n - 1))$]. Values in italics are characteristic of *B. multivorans*, *B. cenocepacia*, or *B. stabilis*, but sometimes similar values are found with other strains. Values in boldface type clearly differentiate individual species or genera.

[d] tr, trace amount (fatty acid was present in an amount lower than 0.1%).

[e] ND, not detected.

[f] Detected as Summed feature 3, which can comprise $C_{15:0}$ iso 2OH, $C_{16:1}$ω7c, or any combination of these fatty acids. Summed feature 3 probably corresponds to $C_{16:1}$ω7c, as this fatty acid has been reported for *Burkholderia* species (2).

[g] Detected as Summed feature 2, which can comprise $C_{14:0}$ 3OH, $C_{16:1}$ iso I, an unidentified fatty acid with an equivalent chain length value of 10.928, $C_{12:0}$ ALDE, or any combination of these fatty acids. Summed feature 2 probably corresponds to $C_{14:0}$ 3OH, as this fatty acid has been reported for *Burkholderia* species (2).

[h] All similarity indexes were calculated as a good library match (0.7 or higher). The similarity index is a software-generated calculation of the distance, in multidimensional space, between the profile of the unknown and the mean profile of the closest library entry. Organisms given could come out as a good library match solely or together as the two closest matches. Apart from individual BCC species, *Pandoraea norimbergensis*, *Pandoraea sputorum*, and *Inquilinus limosum* are not included in the TSBA50 library.

[i] The two isolates in which trace amounts of fatty acid were detected were both from patients with cystic fibrosis.

TABLE 2. Fatty acid compositions of species related to the BCC[a]

Fatty acid[b] or comparison	Fatty acid composition (%)[c] or species identification of:										
	B. gladioli DSM 4285[T]	*P. norimberg.* DSM 11628[T]	*P. apista* CCM 4976[T]	*P. pnomenusa* CCM 4978[T]	*P. pulmonic.* CCM 4979[T]	*P. spurorum* CCM 4980[T]	*R. pickettii* DSM 6297[T]	*C. necator*[i]	*Achromobacter xylosoxidans xylosoxidans*[i]	*Delftia acidovorans*[i]	*I. limosum* DSM 16000[T]
Saturated											
12:0	tr[d] (1)	**2.7 ± 0.2**	**1.8 ± 0.1**	**1.5 ± 0.1**	**2.0 ± 0.1**	**1.7 ± 0.2**	0.1 (1)	n[e]	0.5	2.5	ND[f]
14:0	3.9 ± 0.2	**0.3 ± 0.0**	**0.5 ± 0.1**	**0.7 ± 0.0**	**0.4 ± 0.0**	**0.5 ± 0.1**	4.4 ± 0.2	2.8	2.1	0.7	ND
16:0	24.9 ± 0.8	17.8 ± 0.8	24.7 ± 1.3	21.0 ± 0.8	22.7 ± 0.7	21.3 ± 1.3	26.7 ± 2.1	24.5	34.7	33.2	**1.4**
17:0	0.2 ± 0.1	0.5 ± 0.1	0.2 (1)	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.5 ± 0.1	1.6	n	n	**4.2**
18:0	1.1 ± 0.2	0.9 ± 0.0	0.5 ± 0.1	0.9 ± 0.1	1.0 ± 0.1	0.6 ± 0.1	0.4 ± 0.0	0.4	2.6	n	1.3
Unsaturated											
13:1 AT 12-13	0.5 ± 0.6	0.7 ± 0.6	0.6 ± 0.6	1.2 ± 1.2 (2)	0.7 ± 0.7	0.3 ± 0.3	ND	n	n	n	ND
16:1ω5c	ND	0.3 ± 0.1	0.3 ± 0.1	0.2 (1)	0.3 (1)	0.4 ± 0.1	0.2 ± 0.0 (2)	n	n	n	0.3
16:1ω7c[g]	16.0 ± 4.0	22.4 ± 3.9	23.8 ± 2.1	20.2 ± 1.8	20.8 ± 2.0	13.5 ± 1.4	33.7 ± 1.1	35.5	31.4	40.1	3.4
18:1ω7c	28.0 ± 2.73	33.6 ± 1.6	27.6 ± 1.6	34.1 ± 1.1	34.0 ± 0.8	30.8 ± 4.7	20.2 ± 1.6	22.3	**9.7**	17.2	46.7
Hydroxy											
10:0 3OH	ND	ND	ND	ND	ND	ND	ND	n	n	**2.5**	ND
12:0 2OH	ND	**0.3 ± 0.1**	**0.9 ± 0.0**	**0.8 ± 0.1**	**0.7 ± 0.0**	**1.2 ± 0.1**	ND	n	**2.5**	n	ND
14:0 2OH	ND	ND	ND	0.2 (1)	0.1 (1)	0.1 ± 0.0	0.8 ± 0.3	2.5	2.2	n	ND
14:0 3OH[h]	4.5 ± 0.3	5.7 ± 0.4	5.2 ± 0.0	5.0 ± 0.5	5.4 ± 0.2	5.4 ± 0.0	6.4 ± 0.1	9.2	7.6	n	1.2
16:0 2OH	0.9 ± 0.2	0.2 ± 0.1 (2)	0.2 (1)	0.3 ± 0.1	0.2 (1)	0.5 ± 0.1	0.2 (1)	n	n	n	0.8
16:0 3OH	3.9 ± 0.6	4.1 ± 0.5	4.4 ± 0.6	3.8 ± 1.0	4.5 ± 0.5	4.5 ± 0.2	**0.1 (1)**	**n**	n	n	1.3
16:1 2OH	1.0 ± 0.1	0.4 ± 0.1	0.5 ± 0.2	0.5 ± 0.1	0.3 (1)	0.4 ± 0.2	1.2 ± 0.5	n	n	n	ND
18:1 2OH	1.3 ± 0.2	0.8 ± 0.2	0.8 ± 0.2	1.8 ± 0.5	0.6 ± 0.1	1.9 ± 0.4	3.0 ± 0.6	n	n	n	15.9
Cyclo											
17:0 cyclo	8.5 ± 3.1	6.1 ± 3.3	6.2 ± 2.0	5.6 ± 0.8	4.2 ± 1.5	10.8 ± 3.0	**0.8 ± 0.2**	**n**	5.6	3.3	ND
19:0 cyclo ω8c	5.1 ± 3.1	2.7 ± 1.6	2.0 ± 1.0	2.5 ± 0.3	2.1 ± 1.0	5.4 ± 2.0	**ND**	**n**	n	n	9.6
Library comparison											
TSBA50[j]	*B. gladioli*/ *B. cepacia*	*B. pyrrocinia*/ *P. pnomenusa*	*P. apista*	*P. pnomenusa*	*P. pnomenusa*/ *P. pulmonicola*	*B. cepacia*	*R. pickettii*/ *Erwinia chrysanthemi* biotype II	Library match	Library match	Library match	No match

[a] Fatty acid compositions of BCC type strains are given in Table 1. Fatty acids present in all species in an amount lower than 0.5% were omitted. Cultivation conditions for the TSBA50 standard library were used.

[b] In a designation, the first value is number of C atoms in the fatty acid. The number following the colon is the number of double-bound C atoms. The value following the letter ω signifies the position in the carbon chain (counted from opposite the carboxyl group). The number preceding OH indicates the number of OH groups. cyclo, cyclopropane circle; c, *cis* conformation.

[c] Values are means ± standard deviations. Values in parentheses show how many times the fatty acid appeared. Mean values were calculated either for three independent analyses (type strains) or for several isolates (one isolate each for *B. multivorans*, *B. cenocepacia*, and *B. stabilis*) by using FAME data from the standard MIS Sherlock composition report. With the aid of MS Excel software, the standard deviations were calculated according to the formula [sgrt $(\Sigma(x - x')^2/(n - 1))$]. Values in boldface type clearly differentiate individual species or genera.

[d] tr, trace amount (fatty acid was present in an amount lower than 0.1%).

[e] n, not mentioned.

[f] ND, not detected.

[g] Detected as Summed feature 3, which can comprise $C_{15:0}$ iso 2OH, $C_{16:1}$ω7c, or any combination of these fatty acids. Summed feature 3 probably corresponds to $C_{16:1}$ω7c, as this fatty acid has been reported for *Burkholderia* species (2).

[h] Detected as Summed feature 2, which can comprise $C_{14:0}$ 3OH, $C_{16:1}$ iso I, an unidentified fatty acid with an equivalent chain length value of 10.928, $C_{12:0}$ ALDE, or any combination of these fatty acids. Summed feature 2 probably corresponds to $C_{14:0}$ 3OH, as this fatty acid has been reported for *Burkholderia* species (2).

[i] Data were taken from the TSBA50 library of MIS Sherlock. Data for *Cupriavidus necator* are included in the library as *Ralstonia eutropha*.

[j] All similarity indexes were calculated as a good library match (0.7 or higher). The similarity index is a software-generated calculation of the distance, in multidimensional space, between the profile of the unknown and the mean profile of the closest library entry. Organisms given could come out as a good library match solely or together as the two closest matches. Apart from individual BCC species, *Pandoraea norimbergensis*, *Pandoraea sputorum*, and *Inquilinus limosum* are not included in the TSBA50 library.

log phase of growth, was harvested. For the CLIN50 library, strains were grown on trypticase soy broth (BBL, Becton Dickinson) solidified with agar (Difco, Becton Dickinson) and 5% sheep blood for 24 h at 35°C; for the TSBA50 library, strains were grown on trypticase soy broth solidified with agar for 24 h at 28°C. From our experience, some BCC strains (*B. ambifaria* type strain and some *B. cenocepacia* clinical isolates) are not able to grow sufficiently after 24 h (in all four quadrants). These strains may require a longer period to obtain sufficient growth in comparison with other members of the BCC. To see how much it can influence the fatty acid results, we prolonged cultivation conditions for the TSBA50 library up to 48 h. All incubations were performed in ambient air.

As with other commercially available BCC differentiation systems, the usefulness of standard libraries included in MIS Sherlock software is limited. From nine species belonging to the BCC, it offers entries of only *B. cepacia* (CLIN50 and TSBA50 libraries) and *B. pyrrocinia* (TSBA50 library). Generally, application of both libraries is appropriate for differentiation of phenotypically related *R. pickettii* and *Pandoraea* spp. from BCC. The TSBA50 comprises a wider database supplemented with more recently described new species than the CLIN50 library, so the TSBA50 library is more favorable for the standard library comparison than the CLIN50. Also, the addition of blood to the CLIN50 standard medium enhances the variability of the fatty acid patterns. After the comparison of fatty acid profiles with the TSBA50 library, all BCC strains were identified, mostly as *B. cepacia*, *B. gladioli*, or *B. pyrrocinia* (Tables 1 and 2). Interestingly, only the *B. multivorans* type strain was misidentified as *Serratia odorifera* or *Raoultella terrigena*; all other *B. multivorans* clinical isolates were "correctly" recognized as *B. cepacia*. That was probably because the *B. multivorans* type strain showed a larger amount of $C_{16:1}\omega7c$ than of $C_{18:1}\omega7c$ fatty acid, which was just the opposite of results obtained with *B. multivorans* clinical isolates.

The possibility of identifying BCC species was further evaluated by the detailed inspection of FAME profiles. There, such fatty acids which would be characteristic for BCC as a group or even for one particular BCC species and whose amount would be minimally influenced by cultivation conditions were searched. Analysis showed that the chosen incubation period and medium affected mostly the amounts of unsaturated and cyclopropane fatty acids (data not shown). This could be the reason why we did not confirm previously published results regarding *B. ambifaria* (2). In our study, the amounts of the unsaturated $C_{16:1}\omega7c$ and $C_{18:1}\omega7c$ fatty acids and of the cyclopropane fatty acids $C_{17:0}$ and $C_{19:0}\omega8c$ detected in *B. ambifaria* were comparable with amounts detected in other BCC members. Differences can be easily explained by the biosynthesis pathway of the cyclopropane fatty acids. In the stationary phase of growth, a part of the unsaturated fatty acids is transferred into the corresponding cyclopropane fatty acid, i.e., $C_{18:1}\omega7c$ to $C_{19:0}$ cyclo $\omega7c$, by insertion of a methyl group through *S*-adenosylmethionine into the double bond of the fatty acid, leading to the cyclic *cis*-11,12-methylenoctadecanoic acid (lactobacillic acid). The sums of unsaturated fatty acids and the corresponding cyclopropane fatty acids were nearly the same amounts in both studies, and these summed values were independent of the length of cultivation. Identification of BCC to the species level due to the amounts of unsaturated or cyclopropane fatty acids proved to be inappropriate, notably in view of potentially diverse laboratory practices. If insufficient growth of a strain appears, it is recommended to prolong the cultivation period or to change the harvested quadrant. Which possibility will be chosen depends on the laboratory's way of carrying out the standard cultivation.

Saturated $C_{12:0}$, $C_{14:0}$, and $C_{18:0}$ fatty acids were considered better markers because they were stable and did not alter significantly with different cultivation conditions. Based on the quantitative differences of these diagnostic fatty acids, we tried to construct an identification key (Fig. 1). The key was constructed to be able to evaluate FAME profiles gained after all cultivation methods were used.

On the basis of the obtained results, we must rectify the previous assumption about the genera *Burkholderia* and *Pandoraea*. Significant percentages of $C_{12:0}$ are no longer essential for their differentiation (1), because *B. pyrrocinia* contained an amount of $C_{12:0}$ fatty acid comparable with all *Pandoraea* type strains (Tables 1 and 2). $C_{12:0}$ was also detected in *B. ambifaria* (13) and noticed in *B. hospita* (5) and *B. sordidicola* (7). Furthermore, $C_{14:0}$ fatty acid was detected in *B. pyrrocinia* in an amount comparable with amounts detected in all *Pandoraea* species. Thus, the previously described absence of $C_{14:0}$ cannot easily distinguish members of the genus *Pandoraea* from the genera *Ralstonia* and *Burkholderia* (1). In our study, differentiation of *Pandoraea* spp. and BCC from *R. pickettii* was possible due to the presence of $C_{16:0}$ 3OH. For *Pandoraea* spp., the presence of $C_{12:0}$ 2OH, which was not detected in BCC, *B. gladioli*, or *R. pickettii* at all, was typical. Also, the fatty acids $C_{16:0}$ 2OH and $C_{16:1}$ 2OH in *Pandoraea* spp. were commonly below 0.5%, whereas amounts of named fatty acids found in BCC were higher (about 1%) and corresponded with *B. gladioli* or other described *Burkholderia* species (for an example, see reference 7).

According to the proposed identification key, the possibility of differentiating *B. pyrrocinia*, *B. ambifaria*, and *B. vietnamiensis* species was tested by the two-dimensional plot cluster analysis technique (Fig. 2), which uses principal component analysis to separate groups of samples in *n*-dimensional spaces to find relationships among fatty acid profiles (Library Generation Software LGS user's manual, version 4.5; MIDI, Inc., Newark, Del.). According to the created two-dimensional plot, it is obvious that observed differences in the amounts of both $C_{14:0}$ and $C_{18:0}$ fatty acids distinguish *B. pyrrocinia*, *B. ambifaria*, and *B. vietnamiensis* from the remaining BCC species. In the two-dimensional plot, the fatty acid profile comparison was not based on $C_{12:0}$, because this fatty acid was not present in all strains tested. Just the presence of $C_{12:0}$ fatty acid additionally improved the distinction of *B. ambifaria* and *B. pyrrocinia* from other BCC members.

Only three of the most frequently isolated BCC species, *B. multivorans*, *B. cenocepacia*, and *B. stabilis*, were obtained from clinical samples. Some minor fatty acids which will be helpful to differentiate *B. multivorans*, *B. cenocepacia*, and *B. stabilis* from the rest of the BCC were noticed (Tables 1 and 2). *B. multivorans* contained the smallest amount of $C_{18:1}$ 2OH; in *B. cenocepacia*, $C_{14:0}$ 2OH was strikingly often detected; and in *B. stabilis*, a repeated occurrence of $C_{17:0}$ was observed. Because clinical isolates of only three species were obtained,

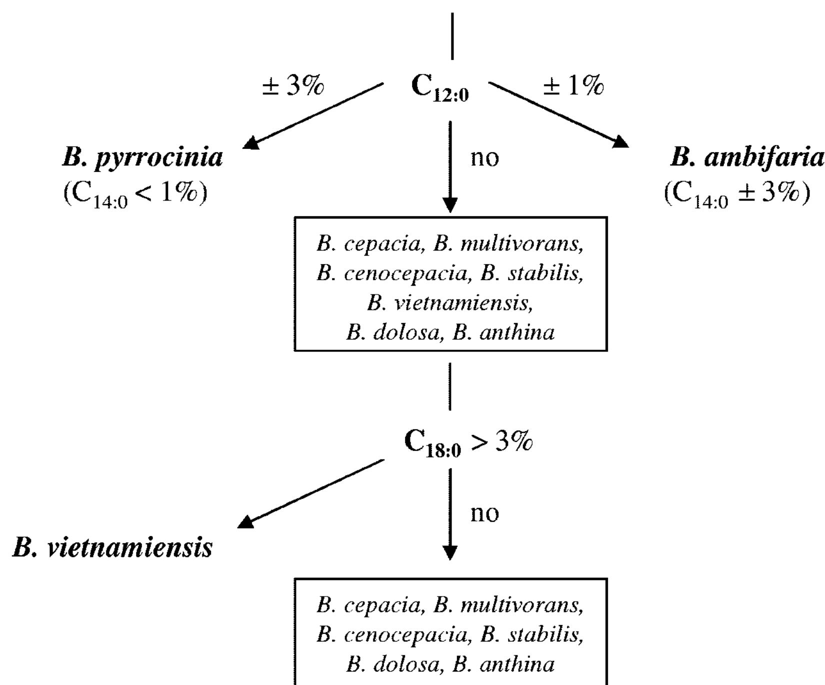

FIG. 1. Key for identification of presumptive BCC isolates according to their fatty acid compositions. Fatty acid compositions obtained by the cultivation of strains along all conditions described (TSBA50 and CLIN50 standard libraries and TSBA50 prolonged up to 48 h) can be evaluated using this identification key. "b" indicates that this composition is also valid for *Cupriavidus* spp. (*C. necator*, *C. gilardii*, *C. pauculus*, *C. taiwanensis*, and *C. respiraculi*) previously found in the respiratory tracts of CF patients (formerly described as *Ralstonia* or *Wautersia*) (see references 3, 11, 16, and 19).

construction of their own library was not practicable. Type strain data are not satisfactory for the judgment if the suggested differentiation of BCC species is valid. To confirm the BCC identification key requires the inclusion of a much wider set of well-characterized strains isolated from various sources and not only from human clinical material (8). To prove our results, testing of a full set of clinical isolates of all BCC species will be needed. Even if *B. pyrrocinia*, *B. ambifaria*, or *B. vietnamiensis* were not present in the tested set of clinical strains studied, their differentiation from other BCC members according to the amount of diagnostic fatty acids was feasible also on the basis of the previously reported results (2, 13, 14).

If there is reason to hesitate about BCC identification, an identification key which offers verification through simple checking of single diagnostic fatty acids could be applied as an additional tool to the standard MIS Sherlock library comparison. FAME analysis could be helpful to confirm *B. ambifaria* or *B. pyrrocinia*, which can generate aberrant 16S rRNA gene-based restriction profiles (17). The appearance of $C_{12:0}$ fatty acid in *recA* restriction fragment length polymorphism types Se6 and AR of *B. pyrrocinia* (10) enables the use of fatty acid analysis as a method of choice for differentiation from *B.*

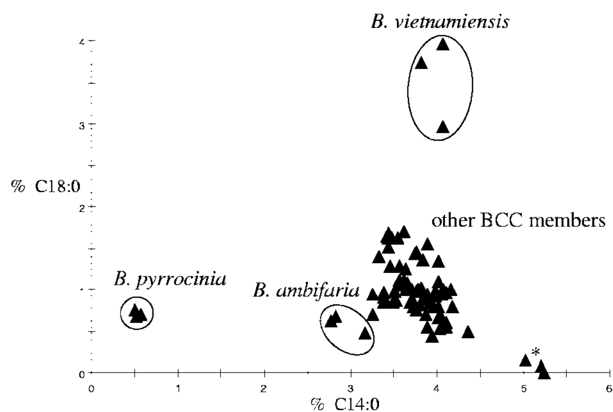

FIG. 2. Relationships among fatty acid profiles depicted in the two-dimensional plot according to the diagnostic fatty acids $C_{14:0}$ and $C_{18:0}$, which were present in all BCC type strains and 47 clinical isolates. The asterisk indicates the *B. multivorans* type strain. For fatty acid profiles used in the two-dimensional plot, strains were cultivated along conditions for the TSBA50 standard library. Reproducibility was ensured by three independent analyses of each strain tested.

cepacia. The two named species can positively react with the same *recA* gene primers (18). These primers were originally designed for *B. cepacia*; a *recA* gene-based PCR assay specific only for *B. pyrrocinia* has not been published yet.

Nevertheless, the differentiation of BCC members to the species level using the identification key is limited; only *B. vietnamiensis*, *B. ambifaria*, and *B. pyrrocinia* could be recognized solely on the basis of the fatty acid analysis. Identification of other BCC members, e.g., the most transmissible species among CF patients, *B. cenocepacia*, *B. multivorans*, and *B. stabilis*, frequently isolated from the respiratory tracts of CF patients must be achieved by more accurate genotypic methods.

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