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Differentiation of *Brevibacterium* spp. Encountered in Clinical Specimens

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Forty-three strains belonging to the genus *Brevibacterium* which were encountered in clinical materials over 2 decades were compared with reference strains, including the type strains, of *B. casei*, *B. epidermidis*, *B. mcbrellneri*, *B. iodinum*, and *B. linens*. By means of carbohydrate assimilation tests (CATs) the 43 clinical isolates could be assigned to the species *B. casei* ($n = 41$) and *B. epidermidis* ($n = 2$). DNA-DNA hybridizations were performed for 20 clinical isolates and confirmed the species identification of the isolates. Cellular fatty acid profiles of all strains were determined and found to have less discriminative power than CATs. This is the first report indicating that most clinical *Brevibacterium* isolates are *B. casei* and that CATs provide an easy-to-perform method for species determination within the genus, thus avoiding nucleic acid techniques.

It has been shown within the last few years that coryneform rods isolated from clinical sources demonstrate much genealogical diversity (9). Among those genera recently recognized in clinical specimens is the genus *Brevibacterium*. After the initial description of the genus *Brevibacterium* by Breed (5), this genus served as a depository for many gram-positive rods later shown by chemotaxonomic investigations to belong to some other genera (7). Today the genus *Brevibacterium* contains only five species, *B. linens* (the type species), *B. casei*, *B. epidermidis*, *B. iodinum*, and the recently defined *B. mcbrellneri* (13, 16). While dairy products and human skin have been described as the habitat of *Brevibacterium* spp. (13, 19), recent reports have emphasized a clinical significance for *Brevibacterium* isolates (6, 12, 15, 18). Species differentiation of these isolates could not be performed since there were no distinguishing phenotypic features or chemotaxonomic characteristics, and nucleic acid techniques were not used. We have developed a simple system using carbohydrate assimilation tests (CATs) to identify clinical *Brevibacterium* isolates to the species level. The results were confirmed by DNA-DNA hybridization studies for 20 of the clinical isolates. CATs proved to be a rapid and reliable method for species determination within this genus.

MATERIALS AND METHODS

Strains. Clinical isolates of *Brevibacterium* spp. which had been collected over 2 decades came from the following culture collections: Department of Medical Microbiology, University of Zürich, Zürich, Switzerland; Laboratoire de Mycologie, University Claude Bernard, Lyon, France; University of Göteborg, Göteborg, Sweden; Laboratory Centre for Disease Control, Ottawa, Canada; and National Collection of Type Cultures, London, United Kingdom. The following reference strains were used in this study: *B. linens* DSM 20425^T (= ATCC 9172^T), *B. linens* DSM 20158 (= ATCC 19391), *B. casei* DSM 20657^T (= ATCC 35513^T), *B. epidermidis* DSM 20660^T (= ATCC 35514^T), *B. epidermidis* DSM 20659 (= ATCC 49089), *B. iodinum* DSM 20626^T (= ATCC 49514^T), *B. iodinum* CIP 59-15 (= ATCC 9897), *B. mcbrellneri* ATCC 49030^T, and *B. mcbrellneri* E2C1 (16). The isolates had been assigned to the genus *Brevibacterium* on the basis of previous chemotaxonomic investigations, i.e., the presence of *meso*-diaminopimelic acid, the absence of mycolic acids, G+C content, and menaquinone patterns (6, 12, 13, 19).

Cultivation of strains. The strains were cultured on Columbia agar (Becton Dickinson, Cockeysville, Md.) supplemented with 5% defibrinated sheep blood in a 5% CO_2 atmosphere at 37°C, except for the *B. linens* strains, which were grown at 25°C because of their lower optimal growth temperature (13). Because of abundant growth, cultures could be harvested after 24 h and processed further.

Assimilation tests. A bacterial suspension was made in 1 ml of 0.85% NaCl to a turbidity of McFarland standard 1 (corresponding to 3×10^8 cells per ml) as measured with an ATB 1550 densitometer (api Biomérieux, Marcy l'Etoile, France). The bacterial suspension was transferred into 15 ml of AUX medium (ammonium sulfate, 2.0 g; agar, 1.5 g; mineral base, 82.8 mg; amino acids, 250.0 mg; vitamins and nutritional substances, 35.9 mg; and phosphate buffer, 0.04 M [pH 7.1], to make 1,000 ml with a final pH of 7.0 to 7.2) (api Biomérieux) and mixed thoroughly. Approximately 300 μl of this suspension was transferred into each cupule of the API 50CH system (api Biomérieux). Assimilation tests were read after 48 and 120 h of incubation at 37°C, with the exception of *B. linens*, which was incubated at 25°C. An opaque cupule indicated a positive reaction, i.e., bacterial growth. Cupules with no bacterial growth remained clear.

CFAs. Cells were grown on Trypticase Soy agar (Becton Dickinson) plates supplemented with 5% sheep blood for 24 h in 5% CO_2 at 37°C, except for *B. linens* which was, again, grown at 25°C. Cellular fatty acid (CFA) patterns were determined by gas-liquid chromatography as outlined previously (22).

DNA-DNA hybridizations. Lysis of cells was achieved using lysozyme and achromopeptidase (Sigma Chemical Co., St. Louis, Mo.); DNA was extracted and purified as described previously (3). Native DNA was labelled by nick translation with ^{3}H-labelled nucleotides (Amersham, Aylesbury, United Kingdom) at a specific activity of 6×10^6 cpm/μg. The hybridization method (S1 nuclease-trichloroacetic acid method) has been described previously (11). The reassociation temperature

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TABLE 1. Origins of the strains studied

Organism (no. of strains studied)	Source or diagnosis	No. of strains
B. casei (42)	Blood culture	19
	Normally sterile body sites (cerebrospinal fluid, eye, knee joint, hip joint [twice], peritoneal dialysate [four times], Douglas pouch, pancreas, intravascular catheter tip, lumbar vertebrae, and pleural fluid)	14
	Suppuration or abscess	2
	Respiratory specimens	2
	Human skin	2
	Corneal ulcer	1
	Toe webs—tinea pedis	1
	Cheese (strain DSM 20657^T)	1
B. epidermidis (4)	Human skin (three times)	3
	Pleural fluid	1
B. mcbrellneri (2)	Genital hair—white piedra	2
B. iodinum (2)	Milk	1
	Unknown	1
B. linens (2)	Cheese	1
	Unknown	1

TABLE 2. Differentiation of *Brevibacterium* spp. by assimilation tests

Substrate	% of strains able to utilize the substrate after 48 (120) h of incubation[a]			
	B. casei (n = 42)	*B. epidermidis* (n = 4)	*B. iodinum* (n = 2)	*B. linens* (n = 2)
Glycerol	100 (100)	100 (100)	50 (50)	50 (50)
Erythritol	0 (0)	0 (0)	0 (0)	50 (50)
D-Arabinose	100 (100)	0 (0)	0 (0)	0 (0)
Ribose	0 (7)	0 (50)	0 (0)	0 (0)
D-Glucose	100 (100)	100 (100)	50 (100)	50 (100)
D-Fructose	98 (98)	100 (100)	50 (100)	50 (100)
D-Mannose	12 (19)	100 (100)	50 (50)	50 (50)
L-Sorbose	0 (0)	50 (75)	0 (0)	50 (50)
Mannitol	0 (0)	100 (100)	0 (0)	0 (0)
N-Acetylglucosamine	86 (88)	0 (0)	0 (0)	0 (0)
Maltose	93 (93)	0 (0)	0 (0)	0 (0)
Saccharose	95 (98)	0 (0)	0 (0)	0 (0)
Trehalose	98 (98)	0 (0)	0 (0)	0 (0)
D-Turanose	98 (98)	0 (0)	0 (0)	0 (0)
L-Fucose	98 (100)	0 (0)	0 (0)	0 (0)
D-Arabitol	0 (0)	100 (100)	0 (0)	0 (0)
Gluconate	100 (100)	100 (100)	0 (0)	0 (0)

[a] *B. mcbrellneri* strains (n = 2) were unable to use any of the substrates after 48 or 120 h of incubation.

was 70°C. DNA-DNA hybridization experiments were carried out using labelled DNA from *B. casei* (DSM 20657^T) and *B. epidermidis* (DSM 20660^T) as probes. DNA homology values were expressed as the percentage of labelled DNA pairing with heterologous DNA relative to the DNA pairing with homologous DNA. Readings were done at least in duplicate, and the median was calculated. The unstandardized reassociation values for homoduplexes ranged from 77 to 85%. The levels of reassociation in control tubes containing only labelled DNA and salmon sperm DNA ranged from 5 to 15%.

RESULTS AND DISCUSSION

Table 1 lists the sources or diagnoses of the 52 strains investigated. Forty-three strains came from clinical material, with blood cultures (44%) or material from other sterile body sites (33%) predominating. The reference strains of *B. casei*, *B. epidermidis*, *B. iodinum*, and *B. linens* came from sources with no clinical relevance. Other authors have recovered brevibacteria mainly from human skin (1, 14, 16, 19), but some newer reports have described *Brevibacterium* spp. in blood cultures, in dialysates, or as the causative agent of osteomyelitis (6, 12, 18).

For the identification of gram-negative, nonfermenting bacteria, CATs have been demonstrated to be of great value (2). These tests are included in the commercial API 20NE system (api Biomérieux). Initial experiments with the 12 assimilation tests contained in this identification system did not clearly allow differentiation among *Brevibacterium* spp. Therefore, we applied the AUX medium (attached to the API 20NE system for assimilation testing) to the API50 CH kit, allowing the observation of the assimilation of 49 carbohydrates (cupule 1 is the negative control). The results of the CATs are shown in Table 2. We found that 41 of 43 (95%) clinical isolates belonged to *B. casei* whereas only 2 of 43 (5%) strains were *B. epidermidis*. None of the 43 clinical strains was found to be *B. mcbrellneri*, *B. iodinum*, or *B. linens*. These findings were confirmed by DNA-DNA hybridizations: 18 representatives of the strains assigned to *B. casei* on the basis of CATS were randomly chosen and found to exhibit DNA relatedness with *B. casei* DSM 20657^T ranging from 73 to 94%. This is in agreement with the species definition proposed by Wayne et al. (24). Under the same conditions, the *B. epidermidis* strains (two reference strains and two clinical isolates) showed a DNA relatedness of 30 to 49% with *B. casei* DSM 20657^T. When using the type strain of *B. epidermidis* (DSM 20660^T) as a probe, we observed 82 to 90% DNA homology with the strains assigned to *B. epidermidis* but only up to 20% DNA relatedness with the 19 *B. casei* strains tested (1 reference strain and 18 clinical isolates).

B. casei utilized more carbohydrates than the other *Brevibacterium* spp. (Table 2). The assimilation of D-arabinose, *N*-acetylglucosamine, maltose, saccharose, trehalose, D-turanose, and L-fucose by *B. casei* and the inability of the other *Brevibacterium* spp. to utilize these carbohydrates provided a clear-cut distinction for the recognition of *B. casei*. Only *B. epidermidis* strains were able to utilize mannitol or D-arabitol. *B. linens* and *B. iodinum* were found to utilize only a few carbohydrates, whereas we did not observe any utilizations by *B. mcbrelleneri*. None of the strains tested utilized any of the following carbohydrates: L-arabinose, D-xylose, L-xylose, adonitol, β-methyl-xyloside, rhamnose, dulcitol, sorbitol, α-methyl-D-mannoside, α-methyl-D-glucoside, amygdaline, arbutine, salicine, cellobiose, lactose, melibiose, inulin, D-raffinose, glycogen, xylitol, β-gentiobiose, D-lyxose, D-tagatose, D-fucose, L-arabitol, and 5-keto-gluconate. Assimilation reactions for the following carbohydrates were not of any discriminative value: galactose, inositol, melezitose, starch, and 2-keto-gluconate. For nearly all strains tested, most of the utilizations could be read after 48 h of incubation. In *B. epidermidis*, the assimilation of ribose became positive only after 120 h of incubation. On the basis of these results we suggest an incubation period of 48 h for CATs.

CFA patterns are of discriminative value for many taxa of gram-positive rods (4, 22). Therefore, we considered their determination for species differentiation within the genus *Brevibacterium*. The CFA patterns of the 52 strains studied are

TABLE 3. CFA profiles of *Brevibacterium* spp.

Component (FAME)	% of CFAs[a] (mean ± SD) in:				
	B. casei (n = 42)	*B. epidermidis* (n = 4)	*B. mcbrellneri* (n = 2)	*B. iodinum* (n = 2)	*B. linens* (n = 2)
i-$C_{15:0}$	8 ± 3	5 ± 1	6 ± 1	6 ± 1	7 ± 1
a-$C_{15:0}$	43 ± 7	62 ± 9	44 ± 1	71 ± 1	62 ± 1
i-$C_{16:0}$	6 ± 2	3 ± 1	12 ± 1	2 ± 0	4 ± 2
$C_{16:0}$	1 ± 1	1 ± 0	4 ± 1	1 ± 0	1 ± 0
i-$C_{17:0}$	3 ± 1	1 ± 0	1 ± 0	1 ± 1	1 ± 0
a-$C_{17:0}$	39 ± 8	27 ± 9	30 ± 1	18 ± 1	21 ± 3

[a] FAMEs with less than 1% were not reported.

shown in Table 3. 12-Methyltetradecanoic (a-$C_{15:0}$) fatty acid methyl ester (FAME) and 14-methylhexadecanoic (a-$C_{17:0}$) FAME were the dominant CFAs for all *Brevibacterium* species. For each of the 52 strains the amount of a-$C_{15:0}$ plus a-$C_{17:0}$ was greater than 75% of all FAMEs (data not shown). This feature can be of value in the differentiation of *Brevibacterium* spp. from other gram-positive, nonfermenting genera (e.g., the genus *Arthrobacter*) (10) which usually contain lower levels of these two FAMEs. *B. epidermidis* was found to have larger amounts of a-$C_{15:0}$ than *B. casei* ($P < 0.05$). This observation might be of some value for the differentiation of *Brevibacterium* spp. even though the data base seems to be quite limited for *B. epidermidis*. Regarding this fact, we asked different type culture collections from all over the world for *B. epidermidis* strains but found only the two isolates (NCDO 2285 and NCDO 2286) initially described by Collins et al. (8).

For the routine laboratory we recommend the following procedure when *Brevibacterium* spp. are suspected to have been isolated from clinical specimens: phenotypically, the distinctive cheese-like smell should raise the suspicion of *Brevibacterium* spp. Colonies are usually whitish grey, with *B. epidermidis* strains becoming slightly yellowish with time. Gram stains show relatively short rods, and a rod-coccus cycle is observed on suitable media (13). As a rapid confirmation test for *Brevibacterium* spp. we recommend methanethiol production (20, 21), but within 2 h, as this reaction also becomes positive for many other genera, e.g., the genus *Bacillus* or *Corynebacterium*, within 4 or 24 h, respectively (23). If available, the CFA profile could confirm the assignment to the genus *Brevibacterium* (see above). When species identification is required, carbohydrate utilizations could be applied as outlined above.

Our data indicate that the clinical microbiologist has to expect *B. casei* when isolating *Brevibacterium* spp. from clinical material. This finding contradicts a previous report which suggested *B. epidermidis* as the majority of clinical *Brevibacterium* isolates (14). It is unclear how Kates et al. (14) and McCaughey and Damani (17) diagnosed *B. epidermidis* with their methods. Their isolates may have been tentatively named *B. epidermidis* since this species was expected from clinical material after the description of Collins et al. (8). On the basis of our results for *Brevibacterium* typing we consider *B. casei* not only to be found on cheese (8) but also to be part of the human skin flora (where it might contribute to the malodor of some people's feet). *B. mcbrellneri* has been isolated only from patients with white piedra so far (16). We are not aware of any instance in which *B. iodinum* was isolated from clinical specimens. *B. linens* might remain undiagnosed in clinical material because of its optimal growth temperature of 25°C.

In our hands CATs were an easy-to-perform test system which yielded fast and reliable results for species differentiation within the genus *Brevibacterium*, thus avoiding nucleic acid methods. Finally, further case reports are needed to make the clinical microbiologist more aware of encountering *Brevibacterium* spp. in clinical specimens.

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