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Characterization of *Brevibacterium* spp. from Clinical Specimens

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Nonfermenting coryneform bacteria identified as *Brevibacterium* spp. were isolated from routine clinical specimens. Four strains were derived from peritoneal fluid and had presumably been involved in the pathogenesis of continuous ambulatory peritoneal dialysis peritonitis. Another five isolates most probably represented skin contaminants. Cell wall and lipid analyses confirmed the genus identification. Strains in this taxon are difficult to distinguish from other biochemically inactive and nonmotile coryneform species but show characteristic cellular fatty acid profiles. In vitro susceptibilities to commonly used antibiotics were determined.

Four species of the genus *Brevibacterium* are recognized in *Bergey's Manual of Systematic Bacteriology* (9): *Brevibacterium linens* (the type species), *B. iodinum*, *B. casei*, and *B. epidermidis*. *B. linens* and *B. casei* are found on surface-ripened cheeses, while *B. iodinum* has been isolated only from milk. *B. epidermidis* forms part of the resident flora of the human skin (9).

Brevibacteria are short, nonbranched, asporogenous, obligately aerobic, gram-positive rods which may exhibit a marked rod-coccus cycle when cells become older. *B. casei* and *B. epidermidis* show optimum growth between 30 and 37°C (which may suggest human or animal origin), whereas *B. linens* and *B. iodinum* prefer temperatures between 20 and 30°C. Members of this genus are nonmotile and salt tolerant (>6.5% NaCl) and produce catalase and proteinases but do not possess urease and fail to produce acid from glucose or other carbohydrates in peptone media. All of the *Brevibacterium* spp. tested so far (*B. linens*, *B. epidermidis*, and *B. casei*) produce methanethiol (CH_3SH) from L-methionine (9). The cell walls of *Brevibacterium* spp. contain *meso*-diaminopimelic acid (*m*-DAP) but no mycolic acids and no arabinose (9). The G+C content of the DNA varies from 60 to 67 mol%. All *Brevibacterium* spp. have similar fatty acid profiles, with anteiso-C_{15} and anteiso-C_{17} acids as major compounds (9).

Except in one report (12), brevibacteria have not been described as human pathogens. Isolates from clinical specimens have been interpreted as skin flora (16). In this report, we present nine strains which have been isolated or received within 1 year in our diagnostic laboratory. During that year, particular attention had been drawn to the identification to species and clinical relevance of coryneform organisms. All strains identified in the context of this study as *Brevibacterium* spp. were characterized biochemically and chemotaxonomically and tested in vitro for antimicrobial susceptibility.

MATERIALS AND METHODS

Strains. Five strains had been isolated from routine diagnostic specimens on Columbia agar (Becton-Dickinson, Basel, Switzerland) supplemented with 5% defibrinated sheep blood (SBA) in ambient air with 5% CO_2 at 37°C. Four were submitted from foreign laboratories for identification (V04 28424, V09 21397, V09 21398a, and V09 21398b; Table 1). Three reference strains, *B. epidermidis* DSM 20659, *B. casei* DSM 20657, and *B. linens* DSM 20158, were furnished by the German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany. Strains DSM 20659 and DSM 20657 are identical to NCDO 2285 and NCDO 2048, respectively (3).

Cultivation of strains. Strains were grown at 37°C on SBA in an aerobic atmosphere. Cellular morphology was observed in Gram-stained smears made from cultures incubated at 37°C for up to 6 days in thioglycollate medium without indicator 135-C (Becton-Dickinson) but with 30 ml of Fildes enrichment (Becton-Dickinson) per liter.

Biochemical characteristics. Except for media used to test for production of methanethiol, DNase, gelatinase, pyrrolidonyl peptidase, and L-alanine aminopeptidase, media were prepared in accordance with reference 13. For rapid detection of methanethiol production, the method of Pitcher and Malnick (16) was used and slants were inspected at 24 h. Diffusion of a bright yellowness through the medium was read as a positive result. Other coryneform bacteria tested for this property were three strains of Centers for Disease Control (CDC) group ANF-1; two strains each of *Rothia*

TABLE 1. Origins of the clinical isolates studied

Laboratory no. of strain	Specimen	Disease	Patient sex[a], age (yr)
V06 18454	Pleural fluid[b]	Lung cancer	M, 72
BLU 2425	Blood[c]	Cholangitis	F, 79
V05 9475	Sputum[d]	Cystic fibrosis	M, 27
V04 28424	Dialysate	CAPD peritonitis	F, 63
V09 22469	CSF[b,e]	Meningitis	M, 45
V10 1863	Fluid from Douglas pouch[b]	Salpingitis	F, 23
V09 21397	Dialysate	CAPD peritonitis	M, 78
V09 21398a	Dialysate	CAPD peritonitis	M, 79
V09 21398b	Dialysate	CAPD peritonitis	M, 79

[a] M, male; F, female.
[b] Single finding after several days of incubation; did not correlate with disease.
[c] One of three blood cultures positive (aerobic bottle only).
[d] Brevibacteria were isolated together with *Pseudomonas aeruginosa* and *C. diphtheriae mitis* var. belfanti.
[e] CSF, cerebrospinal fluid.

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TABLE 2. Biochemical characteristics of nine clinical isolates and reference strains

Characteristic	No. of strains positive (%)	Result[a] obtained with:		
		B. epidermidis DSM 20659	*B. linens* DSM 20158	*B. casei* DSM 20657
Methanethiol	9 (100)	+	+	+
Motility (hanging drop)	0	−	−	−
Catalase	9 (100)	+	+	+
Oxidase (tetramethylphenylenediamine)	0	−	+	−
Urease (Christensen)	0	−	−	−
Esculin hydrolysis	0	−	−	−
Gelatin hydrolysis[b]	9 (100)	+	+	+
Nitrate to NO_2	0	+	−	−
Nitrate to gas	0	−	−	−
TSI[c] agar (slant K/butt NC)[d]	9 (100)	+	+	+
Acid from carbohydrates[e]	0	−	−	−
Hippurate[f]	9 (100)	+	+	+
DNase	9 (100)	+	+	+
Voges-Proskauer	0	−	−	−
Growth at 42°C	4 (44)	+	−	+
Growth in 6.5% NaCl	100	+	+	+
Starch hydrolysis	0	−	−	−
Tyrosine hydrolysis[g]	100	+	+	+
Casein hydrolysis[g]	100	+	+	+
Xanthine hydrolysis[g]	100	+	+	+
CAMP test with *Staphylococcus aureus* ATCC 25923	0	−	−	−
String formation in 3% KOH	0	−	−	−
L-Aminopeptidase	1 (11)	−	−	−
Pyrrolidonylpeptidase	3 (33)	−	−	−
Pyrrolidonylpeptidase[h]	4 (44)	−	−	+
Pyrazinamidase[h]	7 (78)	+	+	+
Alkaline phosphatase[h,i]	9 (100)	+	+	+
α-Glucosidase[h]	8 (89)	−	−	+
α-Glucosidase[i]	8 (89)	−	−	+
C4 esterase[i]	9 (100)	+	+	+
C8 lipase esterase[i]	9 (100)	+	+	+
C14 lipase[i]	2 (22)	−	−	−
Leucine aminopeptidase[i]	9 (100)	+	+	+
Trypsin[i]	4 (44)	−	−	+
Acid phosphatase[i]	8 (89)	+	−	+
Phosphoamidase[i]	8 (89)	+	−	+

[a] +, positive; −, negative.
[b] Positive within 5 days.
[c] TSI, triple sugar iron.
[d] K, alkaline; NC, no change.
[e] 1% of glucose, maltose, sucrose, mannitol, and xylose, respectively, in CTA media (5).
[f] Five clinical isolates and the reference strains were only weakly positive.
[g] Casein and xanthine positive within 5 days, tyrosine positive within 14 days.
[h] API Coryne system.
[i] API ZYM system.

dentocariosa, *Bacillus* species, and *Listeria monocytogenes*; and one strain each of *Corynebacterium diphtheriae mitis*, *C. xerosis*, *C. urealyticum*, *Actinomyces pyogenes*, *C. diphtheriae gravis* (ATCC 19409), and *C. pseudotuberculosis* (ATCC 19410). DNase production was tested with DNase Test Agar with methyl green (Difco Laboratories, Detroit, Mich.). The presence of gelatinase was tested for by immersing film strips (Diagnostics Pasteur, Marnes-la-Coquette, France) in bacterial suspensions and incubating them for up to 1 week. Pyrrolidonyl peptidase was detected by a rapid colorimetric method using disks impregnated with L-pyrrolidonyl-β-naphthylamide (Wellcome Diagnostics, Dartford, United Kingdom). For detection of L-alanine aminopeptidase, test strips with L-alanine-4-nitroanilide (Diagnostica MERCK, Darmstadt, Germany) were incubated for 10 min in bacterial suspensions. Further enzyme and carbohydrate fermentation activities were assayed by using the API ZYM and API Coryne systems (API bioMérieux SA, La Balme-les-Grottes, France). The techniques used followed the guidelines of the manufacturer. Growth under anaerobic conditions (85% N_2, 10% H_2, 5% CO_2) was checked after incubation at 37°C for 3 days.

Metabolic fatty acids. To detect volatile and nonvolatile acid metabolites from glucose, cultures were incubated for 48 h under aerobic conditions in brain heart infusion broth (Difco) containing 1% glucose. Fatty acid analyses were performed as described by Holdeman et al. (5). For qualitative standards, the following mixtures were used: formic, acetic, propionic, isobutyric, *n*-butyric, isovaleric, *n*-valeric, isocaproic, *n*-caproic, and heptanoic acids (each at 10 mM in water; volatile acids); pyruvic and lactic acids (each at 10 mM); and oxalacetic, oxalic, methyl malonic, malonic, fu-

TABLE 3. API Coryne patterns

API Coryne code (no. of strains)	Results	%id, T[a]
611 000 4: low selectivity (4)	*Brevibacterium* sp.	53.8, 0.80
	CDC group B	24.4, 0.69
	Rhodococcus equi	21.0, 0.69
	CDC group ANF	0.8, 0.45
211 000 4: low selectivity (2)	*Brevibacterium* sp.	51.0, 0.86
	R. equi	48.0, 0.82
	CDC group ANF	0.7, 0.50
011 000 4: low selectivity (2)	*R. equi*	93.1, 0.88
	Brevibacterium sp.	6.7, 0.73
	CDC group ANF	0.2, 0.41
210 000 4: low selectivity (2)	CDC group ANF	79.8, 1.00
	Brevibacterium sp.	18.8, 0.94
	C. urealyticum	0.7, 0.61
411 000 4: low selectivity (1)	*R. equi*	83.7, 0.75
	Brevibacterium sp.	14.6, 0.67
	CDC group B	1.3, 0.43
310 000 4: low selectivity (1)	CDC group ANF	77.4, 0.84
	R. equi	17.2, 0.74
	C. pseudodiphtheriticum	4.3, 0.65

[a] %id, percentage of identification (an estimate of how closely the profile corresponds to the taxon relative to all of the other taxa in the data base); T, T index (an estimate of how closely the profile corresponds to the most typical set of reactions for each taxon).

maric, and succinic acids (each at 5 mM; nonvolatile acids) (all were from Supelco SA, Gland, Switzerland).

Cellular fatty acids (CFAs). Approximately 40 mg (wet weight) of cell mass per strain was harvested from Trypticase soy agar (Becton-Dickinson) plates supplemented with 5% sheep blood. CFA compositions were determined by gas-liquid chromatography as described previously (17). Fatty acid profiles were analyzed by using the Library Generation Software version 3.5 (data bases CLIN and TSBA; MIDI, Newark, Del.).

Other cellular compounds. Analyses of whole-cell hydrolysates for *m*-DAP, mycolic acids, and sugars were performed as described by Schaal (18) and Yassin et al. (20).

Antimicrobial susceptibility. Six antibiotics commonly used to treat patients with gram-positive infections were selected for susceptibility testing. MICs of penicillin, erythromycin, clindamycin, tetracycline, vancomycin, and gentamicin were tested by using the National Committee for Clinical Laboratory Standards agar dilution procedure (14). Susceptibility was also determined by using the National Committee for Clinical Laboratory Standards agar diffusion technique on Mueller-Hinton agar with 5% sheep blood (15). Zones of inhibition were recorded in millimeters, since interpretive standards are not available for brevibacteria.

RESULTS AND DISCUSSION

Brevibacteria were isolated from eight individuals (Table 1). Except for one strain (V05 9475), all were isolated in pure culture. In three patients undergoing continuous ambulatory peritoneal dialysis (CAPD) who had peritonitis, the organisms were repeatedly isolated from the peritoneal effluents. Two different isolates (V09 21398a and V09 21398b) came from a single patient. All patients improved quickly during the course of antibiotic treatment. Skin bacteria as opportunistic pathogens are common in CAPD peritonitis (18). The other isolates were thought unlikely to be important in the pathogenesis of infection.

Cells of the nine clinical and three reference strains varied from irregular, slender bacillary forms during the exponential growth phase to coccobacilli in older cultures. The colonies of 10 strains were gray-white on SBA; all were convex with entire edges and were smooth with a shiny surface. They were about 0.5 to 1 mm in diameter after 24 to 48 h of incubation but became larger (2 to 4 mm) after several days. An intensive smell of cheese was detected in all of the strains when the colonies became older. Strains DSM 20158 (*B. linens*) and V06 18454 had a deep orange colony pigmentation that appeared after 48 h of incubation. All of the strains produced diffuse lysis of the sheep blood after 3 days. None of the strains grew under anaerobic conditions.

The biochemical characteristics of test and reference strains are summarized in Table 2. Whenever discrepancies between different test systems were observed for the same reaction, both results are listed. Only the reference strain of *B. linens* was oxidase positive. All strains were negative for β-glucuronidase, β-galactosidase, and *N*-acetyl-β-glucosaminidase and also for esculin, urease, gelatin, and sugar fermenta-

TABLE 4. Main CFAs[a]

CFA	% of total fatty acids in:											
	B. epidermidis	*B. linens*	*B. casei*	V06 18454	BLU 2425	V05 9475	V04 28424	V09 22469	V10 1863	V09 21397	V09 21398a	V09 21398b
i-$C_{15:0}$	4	8	8	5	7	5	3	8	6	9	8	6
a-$C_{15:0}$	71	64	53	68	47	47	50	45	46	42	49	54
i-$C_{16:0}$	2	4	4	4	5	4	5	5	5	5	5	6
a-$C_{17:0}$	18	18	32	22	37	40	39	37	40	40	37	32
MIDI[b] score	0.670[c]	0.650[c]	0.770[c] 0.751[d]	0.801[c]	0.814[d]	0.676[d]	0.595[e] 0.387[d]	0.725[d]	0.682[d]	0.607[f] 0.550[d]	0.876[d]	0.529[d]

[a] Fatty acids of less than 2% are not listed.
[b] TSBA data base. Results obtained with the CLIN data base were "no match" or *Micrococcus* sp. (reference strains of *B. epidermidis* and *B. linens* and strain V06 18454) with scores from 0.188 to 0.420.
[c] *B. epidermidis-linens*.
[d] *B. casei*.
[e] *Microbacterium lacticum*.
[f] *Bacillus coagulans*.

TABLE 5. Antimicrobial susceptibility by agar dilution and disk diffusion technique

Antimicrobial agent (amt in each disk)	MIC (μg/ml), growth inhibition zone around disk (mm)											
	DSM 20659	DSM 20158	DSM 20657	V06 18454	BLU 2425	V05 9475	V04 28424	V09 22469	V10 1863	V09 21397	V09 21398a	V09 21398b
Penicillin G (10 IU)	1, 28	0.5, 30	2, 18	1, 28	0.5, 24	2, 14	2, 19	2, 21	2, 21	2, 20	2, 21	2, 18
Erythromycin (15 μg)	0.5, 25	0.06, 27	0.5, 24	0.125, 23	0.25, 22	1, 21	0.5, 25	0.25, 26	0.5, 19	0.125, 28	0.5, 27	0.125, 28
Clindamycin (2 μg)	4, 20	0.25, 23	0.06, 35	2, 22	1, 15	2, 16	4, 15	4, 17	4, 11	2, 15	4, 13	2, 25
Tetracycline (30 μg)	8, 12	0.5, 27	0.5, 30	0.25, 29	0.25, 26	1, 30	0.5, 29	1, 25	1, 29	0.25, 27	0.25, 30	0.25, 36
Vancomycin (30 μg)	0.12, 22	0.12, 29	0.12, 21	0.125, 23	0.125, 21	0.125, 25	0.12, 26	0.25, 23	0.25, 23	0.125, 22	0.125, 24	0.125, 24
Gentamicin (10 μg)	0.25, 29	0.12, 28	1, 21	0.06, 30	0.5, 28	1, 22	1, 24	0.5, 25	0.5, 25	0.5, 28	1, 25	1, 29

tion reactions in the API Coryne system. They were negative for valine aminopeptidase, cystine aminopeptidase, chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, β-glucosidase, β-glucosaminidase, α-mannosidase, and α-fucosidase in the API ZYM system. Two reference strains, of *B. linens* and *B. epidermidis*, and strain V06 18454 did not produce α-glucosidase in both systems. However, none of the rapid systems unambiguously identified the clinical or reference strains to the species level (Table 3). A review of the API ZYM literature showed no specificity of this enzymatic pattern for brevibacteria (4).

All brevibacteria and the two *Bacillus* sp. strains produced methanethiol from methionine. Representatives of the other genera tested for methanethiol formation were negative, including other obligately aerobic species which were not separable from brevibacteria by the API Coryne or API ZYM system. However, various microorganisms, including nonfermenting gram-negative rods, *Bacillus* spp., anaerobes, yeasts, and filamentous fungi, have been found to produce methanethiol (10). In our laboratory, we have recently isolated two strains of gram-positive oxidative rods that did not belong to any known group but showed the typical CFA patterns of corynebacteria and produced methanethiol. Since only few data (10, 16) are available for gram-positive organisms, the discriminatory power of the methanethiol test for identification of brevibacteria remains unknown.

Analysis of metabolic fatty acids yielded nonspecific identical profiles, as traces (varying from 0.8 to 2.2 mM) of both acetic and lactic acids were produced by all of the strains tested (data not shown). CFAs consisted primarily of anteisomethyl and isomethyl branched acids (Table 4). Quantitative differences were observed in key fatty acids, i.e., 12-methyltetradecanoic (a-$C_{15:0}$) and 14-methylhexadecanoic (a-$C_{17:0}$) acids. The reference strains of *B. epidermidis* and *B. linens* showed larger quantities of a-$C_{15:0}$ but smaller ones of a-$C_{17:0}$ than did the reference strain of *B. casei*. This may be the reason why the MIDI data base TSBA is able to separate the complex *B. epidermidis-B. linens* from the species *B. casei*. Indeed, except for one isolate (V06 18454) that resembled the *B. epidermidis-B. linens* pattern, it identified all clinical isolates with similarity indices of >0.38 as *B. casei* (in two strains as second and third choices, respectively). Other fatty acids found in moderate amounts (<10%) in all strains were isopentadecanoic (i-$C_{15:0}$) and isohexadecanoic (i-$C_{16:0}$) acids. Although the CFA profiles separated our *Brevibacterium* strains into two groups, species-specific CFAs were absent and differences were essentially quantitative. This reduces the discriminatory power of CFAs within this genus, as also observed for other genera of gram-positive rods (1).

Whole-cell hydrolysates of all of the strains tested contained *m*-DAP, glucose, galactose, and ribose but no lysine or mycolic acids. The latter traits clearly distinguish *Brevibacterium* from other actinomycete and coryneform genera, such as *Caseobacter*, *Corynebacterium*, *Gordona*, *Nocardia*, and *Rhodococcus*, which also contain *m*-DAP (7, 9). The sugar patterns obtained by thin-layer chromatography confirmed the presence of glucose, galactose, and ribose in all of the strains tested. As documented by others (11), occurrence of ribose in brevibacteria remains controversial. It was probably derived from nucleic acids and was not a cell wall component. Our data show that the pentose appears, however, to be an intrinsic feature of whole-cell hydrolysates of the strains used in this study. It does not represent a contaminant from the growth medium, since cells had been rigorously washed upon harvesting. Strain V09 21398b contained, in addition, easily detectable amounts of arabinose. This pentose is a characteristic feature of *Corynebacterium*,

TABLE 6. Characteristics that differ between brevibacteria and other nonfermentative, catalase- and gram-positive rods

Organisms	Presence of the following trait[a] (% of strains)[b]:					
	Motility	Urease	Nitrate reduction	Gelatin hydrolysis	Methanethiol production	Other trait(s)
Brevibacterium spp.	−	−	V	+	+	Smell of cheese
CDC group ANF-1	−	−	−	−	−	
CDC group ANF-3	−	−	+ (100)	−	ND[c]	
R. equi	−	+ (76)	+ (43)	−	−	CAMP test positive
C. urealyticum	−	+ (100)	−	+ (47)	−	
C. pseudodiphtheriticum	−	+ (100)	+ (100)	−	−	
Kurthia spp.	+	+ (100)	−	−	ND	
Bacillus spp. (oxidative spp.)	+	V	V	V	V[d]	Spores

[a] +, positive; −, negative; V, variable.
[b] Percentages are from Hollis and Weaver (6).
[c] ND, no data available.
[d] Data available for some *Bacillus* spp. only (10).

Nocardia, *Rhodococcus*, and *Mycobacterium* spp. (7, 18) but has not been observed in brevibacteria (9). Chemotaxonomically, strain V09 21398b closely resembles *C. amycolatum* (2) and *Dermabacter hominis* (8) (cell wall with *m*-DAP, arabinose, and galactose and without mycolic acids). However, these species differ from brevibacteria in being facultatively anaerobic and in producing acid from glucose and other sugars. Furthermore, *C. amycolatum* shows predominantly straight-chain saturated and monounsaturated fatty acids.

The results of agar dilution susceptibility tests were not always in agreement with those of the standard disk diffusion method (Table 5). All strains were susceptible to erythromycin, vancomycin, and gentamicin, while susceptibility to the other antimicrobial agents varied among strains.

Of the four *Brevibacterium* species recognized by Jones and Keddie (9), *B. linens* and *B. iodinum* grow poorly or not at all at 37°C. The former species exhibits an orange pigment, whereas colonies of *B. iodinum* are characterized by production of purple extracellular crystals of iodinin. *B. acetylicum*, a *species incertae sedis*, is fermentative, facultatively anaerobic, and motile by peritrichous flagella (9). It is likely that our clinical isolates belong to *B. epidermidis* and/or *B. casei*, which can be distinguished only by DNA hybridization and the guanine-cytosine ratios of the DNAs (9). The CFA patterns of our isolates were nearly identical to those of the *B. casei* reference strain. Strain V06 18454 is likely to be *B. linens* because of its orange pigment, its CFA profile (closely resembling the *B. epidermidis-B. linens* pattern), and the absence of α-glucosidase (Tables 2 and 4). *B. casei* and *B. linens* have not been reported as belonging to the human skin flora.

In sum, well-growing, strictly aerobic, gram- and catalase-positive, asporogenous rods that are nonmotile and do not ferment sugars may be tentatively identified as *Brevibacterium* spp. on the basis of colony color and morphology (gray-white, opaque, convex, smooth) and an intensive smell of cheese. Distinction from biochemically similar gram-positive rods (6) which may occur in clinical specimens, such as CDC group ANF-3, is possible only by determination of CFAs. Table 6 lists differential features that would separate brevibacteria from other nonfermentative, gram-positive rods by conventional techniques. Methanethiol formation serves to narrow the diagnosis but has been evaluated within a limited number of gram-positive organisms only; therefore, a positive result has to be interpreted with prudence. *Brevibacterium* spp. appear to possess a pathogenic potential for humans and should be included in the list of uncommon organisms in a variety of clinical conditions, such as CAPD peritonitis. In the past, they may have been overlooked or considered apathogenic diphtheroids. As shown by this study, however, they should be distinguished from other coryneform bacteria.

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ADDENDUM IN PROOF

A separation of CDC group B-3 (6) from *Brevibacterium* sp. does not seem possible at this time since morphological and biochemical reactions (including methanethiol) as well as fatty acid composition (1) are identical. Further studies to differentiate these taxa are in progress.

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