Brevibacterium lutescens sp. nov., from human and environmental samples

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Three strains of coryneform rods isolated from clinical samples and one of environmental origin exhibited phenotypic and chemotaxonomic properties characteristic of the genus *Brevibacterium* and their 16S rRNA gene sequences were closely related (98.5–99.0 %) to that of *Brevibacterium otitidis*. However, DNA–DNA hybridization of one strain (CF87^T) showed only 59.6 % relatedness to the type strain of *B. otitidis*, DSM 10718^T, and 75–82 % relatedness to the three other strains. The four strains could be differentiated from *B. otitidis* by cellular fatty acid composition and some phenotypic characteristics. These findings suggest that the four strains belong to a novel species, for which the name *Brevibacterium lutescens* sp. nov. is proposed. The type strain of *B. lutescens* is CF87^T (= DSM 15022^T = CCUG 46604^T).

Recently, a coryneform bacterium belonging to the genus *Brevibacterium* was reported as a cause of peritonitis in a patient undergoing continuous ambulatory peritoneal dialysis (Wauters *et al.*, 2000b). Its conventional phenotypic characteristics were consistent with those of *Brevibacterium otitidis*, and 16S rRNA gene sequencing revealed 98.8 % similarity to the sequence of the type strain of that species, which was rather low, but compatible with membership of the same species (Stackebrandt & Goebel, 1994). Later, three *B. otitidis*-like strains were collected that exhibited a mean of 98.8 % similarity to *B. otitidis* in their 16S rRNA sequences, two from clinical samples and one from the environment. They were studied more extensively along with the peritonitis strain. A few phenotypic characteristics and DNA–DNA hybridization results clearly indicated that the four strains, including the peritonitis strain, belonged to a novel species, closely related to but distinct from *B. otitidis*, for which the name *Brevibacterium lutescens* sp. nov. is proposed.

The four novel strains were of human and environmental origin. Strain CF87^T was isolated from peritoneal fluid (Wauters *et al.*, 2000b), strain CF32 from an infected ear discharge, strain CF60 from a peritoneal dialysate fluid and strain CF100 from a peptone preparation. They were stored

in glycerol at –20 °C and cultured on blood agar at 37 °C in air for the purposes of this study. The following reference strains were included for phenotypic comparison: *Brevibacterium casei* ATCC 35513^T and DSM 9658, *Brevibacterium epidermidis* ATCC 49089, DSM 9585 and DSM 9586, *Brevibacterium mcbrellneri* DSM 9583^T and DSM 9584, *B. otitidis* DSM 10718^T and CCUG 37312, *Brevibacterium iodinum* LMG 2201, *Brevibacterium linens* LMG 3915 and *Brevibacterium paucivorans* DSM 13657^T, DSM 13658, DSM 13659 and DSM 13660. In addition, the following clinical isolates from our collection were included in the study: one *B. otitidis* strain (CF65), confirmed by 16S rRNA gene sequencing and by DNA–DNA hybridization, and eight *B. casei* and seven *B. paucivorans* strains, all of them confirmed by 16S rRNA gene sequencing.

Most phenotypic characteristics were studied as described previously (Funke *et al.*, 1997; Wauters *et al.*, 1998, 2000a, 2001). Pyrrolidonyl arylamidase, α -glucosidase and *N*-acetyl- β -D-glucosaminidase were tested using diagnostic tablets (Rosco). Assimilation/alkalinization of γ -aminobutyric acid was tested using Simmons' citrate agar, replacing citrate by 0.1 % (w/v) of the substrate. Acid production from phenylacetate was examined as follows: 0.1 ml phenylacetate was thoroughly mixed in 100 ml phosphate buffer (0.001 M, pH 7.4) and 0.2 ml of a 1 % (w/v) bromocresol purple solution was added; 0.5 ml of this reagent was mixed with 0.5 ml of a milky suspension (8–9 McFarland) in distilled water obtained from a 24 h culture on TSA. Tubes were incubated overnight at 37 °C and a yellow colour indicated acidification. Carbohydrate assimilation was examined on 50CH strips using AUX medium (bioMérieux).

Abbreviation: CFA, cellular fatty acid.

The GenBank/EMBL/DDBJ accession number for the 16S rDNA sequence of strain CF87^T is AJ488509.

16S rDNA sequence similarities between *B. lutescens* sp. nov. CF87^T and related species are available as supplementary data in IJSEM Online.

API ZYM and API CORYNE strips (bioMérieux) were used according to the manufacturer's instructions.

Antibiotic susceptibility was evaluated by MIC determination using E-test strips (PDM) on Mueller–Hinton blood agar incubated at 37 °C for 24 h. Penicillin, ampicillin, cefotaxime, cephalothin, erythromycin, ciprofloxacin, gentamicin and vancomycin were tested and the results were interpreted according to the criteria established for staphylococci by the NCCLS (2002).

Cellular fatty acids (CFA) were assayed by GLC using a Delsi chromatograph as described previously (Wauters *et al.*, 1996). Amino acid composition of the peptidoglycan was studied by N. Weiss (DSMZ, Braunschweig, Germany) using a TLC method as outlined by Schleifer & Kandler (1972).

For 16S rDNA sequencing, DNA was prepared with Chelex 100 resin (Bio-Rad) as described by O'Neil *et al.* (1996). Briefly, bacteria were lysed by boiling for 15 min in a 5 % Chelex suspension and centrifuged at 13 000 g for 10 min to pellet cell debris. A total of 3 µl of the supernatant containing extracted DNA was used as a template for PCR. The entire 16S rRNA gene (1500 bp) was amplified by PCR using 16S rDNA primers 1522R and 27F (Johnson, 1994). The PCR products were separated by electrophoresis on 1.2 % agarose gel and visualized by ethidium bromide staining under UV. A 1.5 kb fragment was excised from the gel and purified by using a QIAquick gel extraction kit (Qiagen) following the manufacturer's instructions. An ABI PRISM Dye Terminator cycle-sequencing ready reaction kit (Applied Biosystems) was used for sequencing of the PCR product. Sequencing and template preparation were performed in accordance with the instructions of the manufacturer. The universal forward and reverse 16S rDNA sequencing primers 321, 530, 1100, 1242 and 1392 (numbered relative to the *Escherichia coli* 16S rDNA numbering; Johnson, 1994) were used in this study. The sequencing product was purified by ethanol/sodium acetate precipitation (Applied Biosystems) and sequenced with an Applied Biosystems 3100 automatic sequencer. Each 16S rDNA was compared with those available in GenBank and EMBL using the BLAST program. The alignment was created using the CLUSTAL X algorithm (Thompson *et al.*, 1997). For construction of the phylogenetic tree, operations of the PHYLIP package (version 3.5c; J. Felsenstein, Department of Genetics, University of Washington, Seattle, WA, USA) were used. Pairwise evolutionary distances were computed from percentage similarities by the correction of Kimura (1980) and the phylogenetic tree was constructed by the neighbour-joining method (Saitou & Nei, 1987).

DNA–DNA hybridization was carried out by P. Schumann at the DSMZ. DNA was isolated by chromatography on hydroxyapatite (Cashion *et al.*, 1977). DNA–DNA hybridization was performed as described by De Ley *et al.* (1970) with the modification described by Huß *et al.* (1983) and Escara & Hutton (1980), using a Gilford System model 2600

spectrometer equipped with a Gilford model 2527-R thermoprogrammer and plotter. Renaturation rates were computed with the TRANSFER.BAS program (Jahnke, 1982).

On Gram staining, the four strains were coryneform, Gram-positive rods, 2–3 µm long. There was no rod–coccus cycle. Colonies on blood agar reached 1–2 mm diameter after 48 h incubation at 37 °C. They were smooth and yellowish. Aerobic growth occurred at 37, 30 and 20 °C. There was growth on agar containing up to 10 % NaCl. Carbohydrates were not acidified. Urease, nitrate reduction, indole production, decarboxylation of lysine and ornithine and arginine dihydrolase were negative. Gelatin and casein were hydrolysed but tyrosine and xanthine were not. Methanethiol was produced. Pyrazinamidase and pyrrolidonyl arylamidase were positive. There was utilization and alkalization of γ -aminobutyric acid on Simmons' agar base. A buffered phenylacetate solution was acidified. With Rosco diagnostic tablets, α -glucosidase was negative but *N*-acetyl- β -D-glucosaminidase was positive. With the API ZYM system, only lipase (C14), lipase esterase (C8), leucine arylamidase and phosphoamidase were positive in all strains. Acid and alkaline phosphatase were positive in strain CF100 only. Using 50CH strips, no carbohydrates were assimilated. The API Coryne code obtained with strains CF32, CF60 and CF100 was 6102004 and that for strain CF87^T was 6002004. The strains were susceptible to all antibiotics tested except that strain CF87^T was resistant to erythromycin and strain CF100 was resistant to penicillin.

The CFAs were of the branched type, with anteiso C15:0 and anteiso C17:0 being the predominant components, accounting for more than 75 % of the total, as generally observed in *Brevibacterium* (Funke *et al.*, 1997). Although the qualitative composition of the CFAs of the study strains was comparable to that of *B. otitidis*, there were significant quantitative differences in some components, as shown in Table 1. The peptidoglycan was of the A1 γ type, *meso*-diaminopimelic acid being the diamino acid. This is consistent with the genus *Brevibacterium* (Funke *et al.*, 1997).

16S rRNA gene sequencing of strain CF87^T revealed the highest similarity (98.8 %) to *B. otitidis* (Table A, available

Table 1. CFA composition of strains of *B. otitidis* and *B. lutescens* sp. nov.

Values are mean (range) percentages of total CFA. The *B. otitidis* strains tested were DSM 10718^T, CCUG 37312 and CF65.

Fatty acid	<i>B. otitidis</i> (n = 3)	<i>B. lutescens</i> sp. nov. (n = 4)
iso 15:0	17.3 (15.4–21.2)	3.2 (1.9–5.4)
anteiso 15:0	26.8 (24.5–29.1)	43.3 (40.0–50.9)
iso 16:0	17.5 (8.1–20.6)	4.2 (2.5–7.0)
iso 17:0	5.2 (4.3–6.8)	0.5 (0–1.1)
anteiso 17:0	33.6 (31.3–35.3)	40.0 (33.6–50.2)

as supplementary material in IJSEM Online). For the three other strains, 16S rDNA similarity was 98.5–99.0 % to the type strain of *B. otitidis* and 99.3–100 % to CF87^T. In contrast, strain CF65 displayed 99.7 % similarity to *B. otitidis* and only 98.4–98.8 % similarity to the four study strains.

DNA–DNA hybridization between strain CF87^T and *B. otitidis* DSM 10718^T showed only 59.6 % relatedness, whereas strain CF65 showed 84.1 % relatedness to *B. otitidis* DSM 10718^T, consistent with the assignment of strain CF65 to the species *B. otitidis*. Furthermore, levels of relatedness of strains CF32, CF60 and CF100 to strain CF87^T were respectively 77.8, 82.1 and 75.6 %, suggesting that they belong to a single species. The DNA G + C content of strain CF87^T was 68.8 mol%.

Phenotypic, chemotaxonomic and 16S rRNA gene sequencing are consistent with the assignment of the study strains to the genus *Brevibacterium*. They are closely related to *B. otitidis* by 16S rRNA gene sequencing and phenotypic characterization. However, DNA–DNA hybridization clearly suggests that they belong to a distinct species. Some cultural and biochemical characteristics distinguish them from *B. otitidis*. Although colonies of both species displayed a yellow pigment, pigmentation was often visible

Fig. 1. Unrooted tree based on 16S rDNA sequences showing the phylogenetic position of *Brevibacterium lutescens* sp. nov. CF87^T within the genus *Brevibacterium*. Bar, 1 substitution per 100 nt.

sooner in most of the study strains. They grew at a concentration of 10 % NaCl, in contrast to *B. otitidis*. Using Rosco tablets, *N*-acetyl- β -D-glucosaminidase (nitrophenyl conjugate) was positive in the study strains and negative in *B. otitidis*. However, the same enzymic reaction was negative for both species when tested by API CORYNE and API ZYM strips (naphthyl conjugate). Furthermore, the study strains

Table 2. Characteristics that differentiate *B. lutescens* sp. nov. from other *Brevibacterium* species

Species: 1, *B. lutescens* sp. nov. ($n=4$); 2, *B. otitidis* ($n=3$); 3, *B. casei* ($n=10$); 4, *B. epidermidis* ($n=3$); 5, *B. mcbrellneri* ($n=2$); 6, *B. paucivorans* ($n=11$); 7, *B. iodinum* ($n=1$); 8, *B. linens* ($n=1$). +, Positive; –, negative; (+), delayed positive; +/–, most strains positive; –/+, most strains negative; W, weak reaction; V, variable; ND, not determined.

Characteristic	1	2	3	4	5	6	7	8
Colony colour	Yellowish	Yellowish	White-greyish	White-yellow	Greyish	Greyish	Greyish	Yellow
Colony texture	Smooth	Smooth	Smooth	Smooth	Dry, friable	Smooth or sticky	Smooth	Smooth
Growth at/on:								
20 °C	+	–	+	+	–	–	+	+
10 % NaCl	+	–	+	+	–	–	ND	ND
Hydrolysis of:								
Casein	+	+	+	+	+	–	+	+
Gelatin	+	+	+	+	+/(+)	(+) ^w	+	+
Reduction of nitrate	–	–	–/+	+/-	–	–	+	+
Acid from:								
2,3-Butylene glycol*	–	–	–	–	+	–	–	–
Phenylacetate	+	–	+	–	+	+	+	+ ^w
Utilization of:								
D-Arabinose	–	–	+	–	–	–	–	–
Mannitol	–	–	–	+	–	–	–	–
Gluconate	–	–	+	+	–	–	–	–
γ -Aminobutyric acid (Simmons' base)	+	–	+	+	–	–	+	+
α -Glucosidase	–	–	+/-	–	–	–	–	–
<i>N</i> -Acetyl- β -D-glucosaminidase†	+	–	–	+	+	+/-	–	–
Pyrrolidone peptidase	+	+	V	–	–	–	–	–
Pyrazinamidase	+	+	+	+	–	–	+	+

*Determined according to Wauters *et al.* (2001).

†Tested with nitrophenyl compound.

grew at 20 °C within 3 days on blood agar, while *B. otitidis* strains did not, even after 7 days. Utilization and alkalization of γ -aminobutyric acid on Simmons' agar base was positive for the study strains, while the test was negative for *B. otitidis*. The study strains produced acid from phenylacetate using the method described above, while *B. otitidis* strains did not.

There is genetic, chemotaxonomic and phenotypic evidence that strains CF87^T, CF32, CF60 and CF100 belong to a single species within the genus *Brevibacterium*, distinct from the other species described in this genus and most closely related to *B. otitidis* (Fig. 1). The name *Brevibacterium lutescens* sp. nov. is proposed for this novel species. Differential characteristics of *B. lutescens* and other *brevibacteria* are reported in Table 2.

The four strains of *B. lutescens* were isolated from human and environmental sources. Although CF87^T was considered clinically relevant as the causative agent of a peritonitis (Wauters *et al.*, 2000b), further observation is needed to assess the potential pathogenicity of this species and to determine its ecological niche.

Description of *Brevibacterium lutescens* sp. nov.

Brevibacterium lutescens (lu.tes'cens. L. neut. adj. *lutescens* yellowish, because colonies exhibit a yellow pigment).

Cells are Gram-positive, non-motile, non-sporulating rods, 2–3 μ m long, with diphtheroid arrangement. There is no rod-coccus cycle. Colonies are smooth, yellowish and approximately 1–2 mm in diameter after 48 h incubation at 37 °C. Optimal growth occurs between 30 and 37 °C, but strains also grow at 20 °C within 3 days. Growth occurs in 10 % NaCl. No carbohydrates are acidified or assimilated in the API 50CH system. Urease, pyrazinamidase and nitrate are negative. Methanethiol is produced. Gelatin and casein are hydrolysed but tyrosine, xanthine and aesculin are not. Ethylene glycol is acidified. γ -Aminobutyric acid is utilized and alkalized on Simmons' agar base. Phenylacetate is acidified. Pyrrolidonyl peptidase is strongly positive. *N*-Acetyl- β -D-glucosaminidase is positive when the nitrophenyl compound is used as substrate (Rosco). Using the API ZYM system, lipase (C14), lipase esterase (C8), leucine arylamidase and phosphoamidase are positive. Acid and alkaline phosphatase are variable (negative for the type strain). CFAs are of the branched type, with anteiso 15:0 and anteiso 17:0 being the major components. The diamino acid of the peptidoglycan is *meso*-diaminopimelic acid. The DNA G + C content of the type strain is 68.8 mol%.

The type strain, CF87^T (=DSM 15022^T=CCUG 46604^T), was isolated from peritoneal fluid of a patient undergoing dialysis. Three other strains, CF32 (=DSM 15023=CCUG 46605), CF60 (=DSM 15024=CCUG 46606) and CF100 (=DSM 15025=CCUG 46607), were isolated from human clinical samples and from environmental sources.

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