Two Coryneform Bacteria Isolated from the Surface of French Gruyère and Beaufort Cheeses Are New Species of the Genus *Brachybacterium*: *Brachybacterium alimentarium* sp. nov. and *Brachybacterium tyrofermentans* sp. nov.†

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New species names, *Brachybacterium alimentarium* and *Brachybacterium tyrofermentans*, are proposed for two coryneform bacteria isolated from the surfaces of Gruyère and Beaufort cheeses. These two species are similar in their biochemical and chemotaxonomic characteristics but distinct from previously described bacteria. The most distinctive characteristics are the presence of *meso*-diaminopimelic acid-containing peptidoglycan with a D-Glu-D-Asp interpeptide bridge and the presence of erythritol teichoic acids that contain diaminoglucuronic acid (an uncommon substituent). The menaquinone pattern of these organisms is unique among coryneform bacteria. DNA-DNA hybridization experiments revealed that the level of hybridization between the two organisms is 15%, which indicates that they are distinct species. Despite the unique biochemical characteristics of these bacteria, a 16S rRNA sequence comparison revealed that they are unquestionably related to *Brachybacterium faecium*, *Brachybacterium nesterenkovi*, and *Brachybacterium conglomeratum*. DNA-DNA hybridization experiments performed with these three species, *B. alimentarium*, and *B. tyrofermentans* revealed that the levels of complementarity ranged from 11 to 38%, values that are similar to the values obtained for *Brachybacterium* strains described previously. With the inclusion of *B. alimentarium* and *B. tyrofermentans* the genus *Brachybacterium* becomes somewhat heterogeneous with respect to chemotaxonomic characteristics.

Accolas et al. (2) described the isolation of a number of highly salt-tolerant (15 to 20% NaCl) coryneform bacteria from the surfaces of Gruyère and Beaufort cheeses. All of these organisms grew best at temperatures between 20 and 30°C. Most were able to hydrolyze gelatin but not starch and could not decompose uric acid. Deamination of arginine did not occur or occurred only after 1 month (1).

The cell walls of two of the isolates were particularly interesting. These cell walls contained a peptidoglycan type not found in any previously described bacteria and also contained an uncommon erythritol teichoic acid (24). The novel features of these isolates, designated strains CNRZ 925^T (T = type strain) and CNRZ 926^T, made their taxonomic position interesting. The peptidoglycan type of strains CNRZ 925^T and CNRZ 926^T is the *meso*-diaminopimelic acid-D-Asp-D-Glu type and resembles the *meso*-diaminopimelic acid-(D-Glu)₂ type found in members of the genus *Brachybacterium* (6, 11).

We studied a number of taxonomic characteristics of strains CNRZ 925^T and CNRZ 926^T and compared these organisms with strains belonging to the genus *Brachybacterium*.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Strains CNRZ 925^T and CNRZ 926^T were obtained from the Centre National de Recherches Zootechniques (CNRZ), Jouy-en-Josas, France. Reference strain *Brachybacterium conglomeratum* NCIMB

9859 was obtained from the National Collection of Industrial and Marine Bacteria (NCIMB), Aberdeen, United Kingdom, and reference strains *B. conglomeratum* CCM 2136 and *Brachybacterium nesterenkovi* CCM 2432 were obtained from the Czechoslovak Collection of Microorganisms (CCM), Brno, Czech Republic. *Brachybacterium faecium* DSM 4810^T, *B. nesterenkovi* DSM 9573^T, *Arthrobacter globiformis* DSM 20124^T, and *Dermabacter hominis* DSM 7083^T were obtained from the DSM-Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM), Braunschweig, Germany.

The strains were grown aerobically at 30°C in a medium containing (per liter) 10 g of tryptone, 5 g of yeast extract, 5 g of glucose, and 5 g of NaCl (pH 7.2); strains CNRZ 925^T and CNRZ 926^T were also grown in a medium containing (per liter) 17 g of tryptone, 3 g of soy peptone, 5 g of yeast extract, 2.5 g of glucose, 5 g of NaCl, 2.5 g of KH₂PO₄, and 1 ml of Tween 80 (pH 7.3). For solid media 12 g of agar per liter was added.

Morphological and biochemical properties. Cell morphology was studied by using cultures grown for 16 and 48 h. Growth at different pH values and temperatures was examined by using pH values of 5.5 to 9 and temperatures of 20 to 42°C; these experiments were performed in 100-ml Erlenmeyer flasks with integrated cuvettes. Optical density at 580 nm was determined with a Milton Roy spectrophotometer.

Utilization of sugars, tyrosine, and xanthine, NO₃⁻ and NO₂⁻ reduction, and hydrolysis of casein, starch, and gelatin were studied by using the methods of Seiler et al. (25).

Utilization of each of the 20 naturally occurring amino acids (100 mg/liter) was determined on MOPS (morpholinepropanesulfonic acid) medium (21) supplemented with (per liter) 40 mg of adenosine, 40 mg of guanosine, 40 mg of cytidine, 40 mg of thymidine, 40 mg of uridine, 0.1 mg of vitamin B₁₂, 0.1 mg of *p*-aminobenzoic acid, 2 mg of biotin, 2 mg of folic acid, 5 mg of riboflavin, 5 mg of pantothenic acid, 5 mg of lipoic acid, and 10 mg of pyridoxine-HCl. Decreases in the concentrations of single amino acids were determined with an amino acid analyzer (Biotronic, Maintal, Germany).

Production of indole and ammonification were determined by using a medium containing 40 g of peptone per liter. The presence of liberated NH₃ was determined with Nessler's reagent, and the presence of indole was determined with Kovács reagent. Lecithinase activity and tellurite reduction were examined by using a medium containing (per liter) 5 g of meat extract, 10 g of casein peptone, 1 g of yeast extract, 10 g of sodium pyruvate, 12 g of glycine, 5 g of lithium chloride and 15 g of agar (pH 6.8) to which 50 ml of egg yolk emulsion per liter

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† We dedicate this publication to Prof. Dr. Otto Kandler on the occasion of his 75th birthday.

and 3 ml of a 3.5% potassium tellurite solution per liter were added after the medium was cooled to 50°C.

Lipase activity was determined on tributyrin agar containing (per liter) 2.5 g of meat extract, 2.5 g of casein peptone, 3 g of yeast extract, 10 ml of glyceroltributyrate, and 12 g of agar (pH 7.5). Gas production was determined in culture medium by using a Durham tube. Urease production was examined on the urea agar base of Christensen (Merck) to which 5 ml of a 40% urea solution was added after the medium was cooled to 50°C. Denitrification was examined in nitrate broth (Merck). A control medium without KNO_3 was included. Tolerance to NaCl was tested in culture medium containing 0 to 20% NaCl. Susceptibility to antibiotics was tested in culture medium containing penicillin G, cycloserine, chloramphenicol, streptomycin, rifampin, kanamycin, or bacitracin (each at a concentration of 0.5 mg/ml). The presence of catalase was tested by applying 3% H_2O_2 to colonies grown on solid medium. Oxidase activity was determined by using the modified oxidase test of Faller and Schleifer (8).

Chemotaxonomic characteristics. Fatty acids were isolated by the method of Minnikin et al. (19), and fatty acid contents were determined by gas-liquid chromatography as described by Kroppenstedt and Kutzner (15). Menaquinones were isolated by the method of Collins et al. (7) and were identified by high-performance liquid chromatography (HPLC) (14). Cell walls were prepared and total amino acids were analyzed as described previously (24). Phosphorus contents were determined by the method of Ames (3). The erythritol, sugars, and glucosamine in teichoic acids or polysaccharides in the cell walls were analyzed by gas-liquid chromatography as described previously (24). Diaminoglucuronic acid was detected with an amino acid analyzer (Biotronic), and diaminoglucuronic acid contents were determined by using trifluoroacetic acid hydrolysates (2 M trifluoroacetic acid, 3 h, 100°C) or hydrofluoric acid hydrolysates (60% HF, 16 h, 0°C [4]; followed by treatment with 2 M trifluoroacetic acid). The glycolic acid contents of cell wall hydrolysates (4 M HCl, 1 h, 100°C) were determined by cation-exchange HPLC by using an Aminex HPX-87H column and the method described by Guerrant et al. (10).

Sequence determination. DNA purification, in vitro amplification of 16S rRNA-encoding DNA, and direct sequencing of amplified DNA were performed as described previously (26).

Phylogenetic analysis. 16S rRNA sequences were compared with about 1,800 homologous primary structures from bacteria (16, 20) by using the alignment tool of the ARB program package (28). Phylogenetic analyses were performed by using distance matrix, maximum-parsimony, and maximum-likelihood methods

and the corresponding tools of the ARB (28) and PHYLIP (9) program packages, as well as the fastDNAmI program (16). The compositions of the data sets varied with respect to the reference sequences and the alignment positions included. Positional variations at the individual alignment positions were determined by using the tools of the ARB package and were used as criteria to remove or include variable positions.

Isolation of DNA and DNA-DNA hybridization. Chromosomal DNA was isolated from bacterial cells grown to the early stationary phase by using the method of Marmur (17). The DNA purity and concentrations of DNA were determined spectrophotometrically. DNA-DNA hybridization experiments were performed by using DNA labelled with α - ^{35}S -dCTP (Amersham, Little Chalfont, United Kingdom) by nick translation (12). The hybridization experiments were performed on nitrocellulose filters in $2\times$ SSC ($1\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0) at 68°C for 20 h. Nonspecifically bound DNA was removed by stringent washing in $0.1\times$ SSC containing 0.1% sodium dodecyl sulfate for 3 h at 68°C. The DNA reassociation rates were determined at the beginning of saturation (45 μg of DNA) by measuring ^{35}S contents with a scintillation counter (model MR 300; Kontron). DNA from *A. globiformis* DSM 20124^T was used as the negative control.

DNA base composition. The average guanine-plus-cytosine (G+C) molar ratio of DNA was determined by the thermal denaturation method (18). Reference DNA was obtained from *Micrococcus luteus* DSM 20030^T.

Nucleotide sequence accession numbers. The nucleotide sequences of strain CNRZ 925^T, strain CNRZ 926^T, *B. faecium* DSM 4810^T, *B. nesterenkovi* DSM 9573^T, *B. conglomeratum* NCIMB 9859, and *D. hominis* DSM 7083^T have been deposited in the EMBL Data Library under accession numbers X91031, X91657, X91032, X91033, X91030, and X91034, respectively.

RESULTS

Cultural and morphological properties. In the exponential phase of growth strains CNRZ 925^T and CNRZ 926^T were nonmotile, club-shaped rods that occurred in pairs. After 48 h the cells reached the stationary phase and became irregular cocci that occurred in small (CNRZ 925^T) or relatively large (CNRZ 926^T) agglomerations (Fig. 1).

TABLE 1. Characteristics which vary in *Brachybacterium* strains and strains CNRZ 925^T and CNRZ 926^T

Characteristic	Strain CNRZ 925 ^T	Strain CNRZ 926 ^T	Strain DSM 4810 ^T	Strain DSM 9573 ^T	Strain CCM 2432	Strain NCIMB 9859	Strain CCM 2136
Growth	Facultative	Facultative	Aerobic	Facultative	Facultative	Aerobic	Aerobic
Acid produced from glucose anaerobically	—	—	—	+	+	—	—
pH optimum	7.3	7.3	7.3	7.0	7.0	7.3	7.3
Tolerance to NaCl (%)	14	16	8	6	11	13	11
Gelatin hydrolysis	+	+	—	—	+	+	+
Starch hydrolysis	—	—	+	+	+	+	+
Nitrite reduction	—	+	—	—	+	+	+
Urease activity	Inducible	—	—	—	—	—	—
Utilization of amino acids ^a							
Aspartate	—	—	+	—	—	—	—
Glutamate	+	+	—	—	+	+	—
Threonine	—	+	+	+	+	+	—
Serine	—	+	—	+	+	+	+
Glycine	—	+	—	+	+	+	—
Alanine	—	+	+	—	+	+	—
Methionine	—	—	+	—	+	+	—
Histidine	+	+	—	+	—	+	—
Susceptibility to antibiotics ^b							
Chloramphenicol	+	+	+	—	—	+	+
Streptomycin	+	+	+	—	—	—	—
Rifampin	+	+	+	—	—	+	+
Kanamycin	+	+	+	—	+	—	+
Bacitracin	+	+	+	—	+	+	+
G+C content (mol%)	73.0	73.0	69.4 ^c	70.0 ^d	71.2 ^e	71.5 ^e	70.6 ^e

^a Amino acid utilization when a mixture of the 20 naturally occurring amino acids was included in the medium.^b All strains were susceptible to penicillin G and cycloserine.^c Data from reference 6.^d Data from reference 11.^e Data from reference 13.

After 2 days on agar media both strains formed medium-sized, smooth, circular, low-convex colonies with entire margins (diameter, 3 to 5 mm). The colors of the colonies of strains CNRZ 925^T and CNRZ 926^T were vivid lemon and pale flaxen, respectively.

Growth occurred at temperatures above 20°C, and optimal growth occurred at 30°C. No growth occurred at ≥37°C. The doubling times at 30°C in the culture medium used were 1.25 h (CNRZ 925^T) and 2.75 h (CNRZ 926^T).

Biochemical properties. We determined biochemical characteristics of strains CNRZ 925^T and CNRZ 926^T, *B. faecium* DSM 4810^T, *B. nesterenkovi* DSM 9573^T and CCM 2432, and *B. conglomeratum* CCM 2136 and NCIMB 9859. All of these strains exhibited catalase and oxidase activities but not lecithinase activity. None hydrolyzed casein or tributyrin. None utilized tyrosine or xanthine. All reduced nitrate to nitrite. These organisms utilized fructose, sucrose, maltose, and lactose and produced acid but no gas from glucose under aerobic conditions. They did not produce indole. The ammonification test was positive for all strains, and all strains were denitrification negative. All were able to reduce tellurite. Characteristics which could be used to discriminate among the strains are shown in Table 1.

Chemotaxonomic characteristics. All of the strains contained menaquinones in their respiratory chains. The major menaquinone was an unsaturated menaquinone with seven isoprenoid units (MK-7). In addition, minor amounts of MK-8 were present in most strains; the only exception was strain CNRZ 925^T, which contained substantial amounts of MK-8 (Table 2).

The amino acids present in all cell wall hydrolysates were alanine, glutamic acid, and *meso*-diaminopimelic acid (molar ratio, 2:2/3:1). In addition, glycine and amide groups were

found in strains CNRZ 925^T, CNRZ 926^T, DSM 4810^T, and NCIMB 9859. In strains CNRZ 925^T and CNRZ 926^T one of the three glutamate residues was replaced by aspartate. Glycolic acid was present in all strains. The detailed compositions of the peptidoglycan constituents are shown in Table 2.

Galactose and glucosamine were found in the cell walls of most strains; the only exception was strain CCM 2136, whose cell walls lacked glucosamine. In addition, glucose was present in strains NCIMB 9859 and CCM 2136, rhamnose was present in strains DSM 9573^T and CCM 2432, and erythritol and *N,N'*-diacetyl-2,3-dideoxy-2,3-diaminoglucuronic acid were present in strains CNRZ 925^T, CNRZ 926^T, and DSM 4810^T. Phosphorus was present in strains CNRZ 925^T, CNRZ 926^T, and DSM 4810^T. Detailed compositions are shown in Table 2.

We found that iso and anteiso fatty acids predominated in the fatty acid patterns of the strains which we investigated. The major fatty acid was 12-methyltetradecanoic acid (anteiso-C₁₅:0). Other fatty acids (iso, anteiso, and nonbranched fatty acids) occurred in minor amounts (up to 10%). The detailed fatty acid compositions of the strains are shown in Table 3.

Phylogenetic considerations. We determined almost complete 16S rRNA gene sequences for strains CNRZ 925^T and CNRZ 926^T, *B. faecium* DSM 4810^T, *B. nesterenkovi* DSM 9573^T, *B. conglomeratum* NCIMB 9859, and *D. hominis* DSM 7083^T. A matrix of the overall levels of 16S rRNA primary structure similarity for the bacteria which we investigated is shown in Table 4. The 16S rRNA sequence of *D. hominis* was nearly identical to the sequence described by Cai and Collins (5). The closest relatives of strains CNRZ 925^T and CNRZ 926^T were *B. faecium* and *B. conglomeratum*, as indicated by similarity values ranging from 97.1 to 97.5%, whereas *B. nesterenkovi* was less closely related (similarity values, 97.2 and 96.9%). The corresponding similarity values for *D. hominis* and

TABLE 2. Chemotaxonomic characteristics

Strain	Menaquinone composition (%)		Relative amt of cell wall components																
			Amino acids									Sugars and phosphate							
	MK-7	MK-8	Asp	Mur	Glu	Gly	Ala	<i>m</i> -Dpm ^a	GlcN	NH ₃	Glycolic acid ^b	Erythritol	P _i	GlcUANAc ^c	Rham-nose	Gal	Glc	GlcN	
CNRZ 925 ^T	64.4	35.6	1.34	0.83	2.19	1.15	1.96	1	1.28	2.07	+	0.63	1.86	0.21		1		1.13	
CNRZ 926 ^T	91.8	8.2	1.07	0.88	1.95	1.14	1.87	1	1.36	1.83	+	1.61	1.61	0.16		1	0.14	0.53	
DSM 4810 ^T	ND ^d	ND		0.87	2.66	0.98	1.66	1	1.48	0.76	+	0.43	0.41	<0.08 ^e		1	0.37	0.21	
DSM 9573 ^T	ND	ND		0.68	3.13		1.83	1	1.67	0.45	+				0.57	1		0.09	
CCM 2432	99.9			0.79	2.94		1.79	1	2.18	0.37	+				0.70	1		0.19	
NCIMB 9859	90.4	9.6		0.83	3.10	0.99	1.97	1	1.43	1.53	+					1	0.56	0.30	
CCM 2136	90.7	9.3		0.81	2.77	1.15	1.92	1	1.14	1.48	+					1	0.25		

^a *m*-Dpm, *meso*-diaminopimelic acid.^b Qualitative estimate.^c GlcUANAc, *N,N'*-diacetyl-2,3-diamino-2,3-dideoxyglucuronic acid.^d ND, not detected.^e As no commercial *N,N'*-diacetyl-2,3-diamino-2,3-dideoxyglucuronic acid was available, the actual amount could not be determined without investigating of teichoic acid fragments; 80% of the erythritol was unsubstituted (amount of erythritol liberated by HF compared with the total amount of erythritol). The amount of acetate as substituent was not determined.

the brachyacteria were considerably lower (94.1 to 94.8%). All other currently available 16S rRNA sequences of gram-positive bacteria with high DNA G+C contents are less than 94.2% similar to the sequences of the previously described brachyacteria and strains CNRZ 925^T and CNRZ 926^T. The phylogenetic relationships of the organisms which we studied and a selection of related reference organisms are shown in Fig. 2.

DNA-DNA hybridization. The results of DNA-DNA hybridization experiments are shown in Table 5. The DNA-DNA complementarity values for strains CNRZ 925^T and CNRZ 926^T were 10.8 and 17.7% in a reverse experiment. The complementarity values for strain CNRZ 925^T and the previously described brachyacteria which we investigated ranged from 11.4 to 38.1%, and the complementarity values for strain CNRZ 926^T and the previously described brachyacteria ranged from 11.8 to 35.8%. The levels of DNA relatedness between *A. globiformis* DSM 20124^T and the brachyacteria were not significant (0 to 2.4%).

DISCUSSION

The taxonomic position of a number of strains that exhibit peptidoglycan variation A4γ of Schleifer and Kandler (22) has been uncertain. These bacteria have been placed in the genera *Micrococcus* and *Arthrobacter* (11, 22), and on the basis of DNA-DNA hybridization data, Schleifer and Lang (23) placed them in a separate genus, which they did not define further. Later, Collins et al. (6) described the genus *Brachybacterium* for some of these strains. Gvozdyak et al. (11) found that

several strains isolated from dairy products belonged to a new species of this genus, and recently, Takeuchi et al. (29) described three additional species of the genus *Brachybacterium*, bringing the number of species in this genus to five (*B. faecium* Collins, Brown, and Jones 1988, *B. nesterenkovi* Gvozdyak, Nogina, and Schumann 1992, *B. conglomeratum* Takeuchi, Fang, and Yokota 1995, *Brachybacterium paraconglomeratum* Takeuchi, Fang, and Yokota 1995, and *Brachybacterium rhamnosum* Takeuchi, Fang, and Yokota 1995). The main chemotaxonomic characteristic of these species is the occurrence of peptidoglycan variation A4γ [*meso*-diaminopimelic acid-(D-Glu)₂ type] (22). A glycine amide substitution at the α-carboxyl group of D-Glu in the peptidoglycan does not occur in *B. nesterenkovi*. Muramic acid occurs mostly in the glycolylated form instead of the acetylated form (29). In all species, the principal menaquinone is MK-7; the principal fatty acids are anteiso-C_{15:0} and anteiso-C_{17:0} acids. The G+C contents of the DNAs of these species range from 68 to 72 mol%. The main sugars in cell walls are galactose and glucose; in *B. rhamnosum* and *B. nesterenkovi* rhamnose is also found (29).

Like *B. nesterenkovi*, the strains described in this paper were isolated from dairy products (Beaufort and Gruyère cheeses), and they have similar characteristics. The peptidoglycans of these strains are variation A4γ peptidoglycans but differ from the peptidoglycans of the previously described brachyacteria. They contain D-Asp-D-Glu rather than D-Glu₂ as the interpeptide bridge. The α-carboxyl group of D-glutamic acid in the peptide subunit is replaced by glycine amide, as is the case in most previously described brachyacteria. Muramic acid occurs in the *N*-glycolylated form. The principal menaquinone is

TABLE 3. Fatty acid compositions of strains CNRZ 925^T and CNRZ 926^T and other *Brachybacterium* strains

Strain	Fatty acid composition (%) ^a												
	i-C _{14:0}	C _{14:0}	i-C _{15:0}	ai-C _{15:0}	i-C _{16:0}	C _{16:1}	C _{16:0}	i-C _{17:0}	ai-C _{17:0}	C _{18:0}	C _{18:1ω9}	C _{18:1ω7}	C _{19c}
CNRZ 925 ^T			1.0	64.3 ^b	4.1		2.7		5.3	5.7	3.8	5.9	7.2
CNRZ 926 ^T	1.5	1.6		69.1 ^b	4.3	2.5	1.3		3.0	1.9		8.1	6.7
CCM 2432	2.1	3.3	4.5	57.2 ^b	2.9		8.8		12.8	3.1			5.3
NCIMB 9859	8.2	12.4	4.2	15.8	19.9 ^b		17.1		9.2	6.5		4.3	2.4
CCM 2136	0.7	0.6	6.2	37.6 ^b	15.8		4.8	4.1	23.3	2.6		2.0	2.3

^a The abbreviations for fatty acids are illustrated by the following examples: C_{16:0}, hexadecanoic acid; C_{16:1}, hexadecenoic acid; i-C_{15:0}, 13-methyltetradecanoic acid; ai-C_{15:0}, 12-methyltetradecanoic acid; C_{18:1ω9}, *cis*-9,10-octadecenoic acid; C_{19c}, cyclopropanonadecanoic acid.^b Major component.

TABLE 4. Similarity matrix based on 16S rRNA sequences

Organism	% 16S rRNA sequence similarity															
	<i>Brachybacterium alimentarium</i> CNRZ 925 ^T	<i>Brachybacterium tyrofermentans</i> CNRZ 926 ^T	<i>Brachybacterium conglomeratum</i> NCIMB 9859	<i>Brachybacterium faecium</i> DSM 4810 ^T	<i>Brachybacterium nesterenkovi</i> DSM 9573 ^T	<i>Dermabacter hominis</i>	<i>Brevibacterium casei</i>	<i>Brevibacterium linens</i>	<i>Renibacterium salmoninarum</i>	<i>Arthrobacter globiformis</i>	<i>Micrococcus luteus</i>	<i>Rothia dentocariosa</i>	<i>Dermatophilus congolensis</i>	<i>Terrabacter tumescens</i>	<i>Curtobacterium citreum</i>	<i>Clavibacter xyli</i>
<i>Brachybacterium tyrofermentans</i> CNRZ 926 ^T	98.7															
<i>Brachybacterium conglomeratum</i> NCIMB 9859	97.4	97.1														
<i>Brachybacterium faecium</i> DSM 4810 ^T	97.5	97.3	98.1													
<i>Brachybacterium nesterenkovi</i> DSM 9573 ^T	97.2	96.9	96.4	97.1												
<i>Dermabacter hominis</i>	94.8	94.1	94.6	94.3	94.7											
<i>Brevibacterium casei</i>	90.0	90.4	90.4	90.8	90.9	90.1										
<i>Brevibacterium linens</i>	90.1	90.3	90.7	90.3	90.9	90.1	97.1									
<i>Renibacterium salmoninarum</i>	91.3	91.4	91.2	91.3	91.9	91.2	89.6	89.8								
<i>Arthrobacter globiformis</i>	93.6	93.6	93.7	93.6	94.2	92.9	90.8	90.5	95.4							
<i>Micrococcus luteus</i>	92.7	92.7	92.5	92.7	93.1	92.7	90.5	90.0	93.7	95.8						
<i>Rothia dentocariosa</i>	91.2	91.1	91.0	91.0	91.7	91.3	90.3	90.9	92.5	93.9	93.9					
<i>Dermatophilus congolensis</i>	91.5	91.1	91.1	91.1	92.0	91.7	90.0	90.2	91.8	92.5	92.5	92.3				
<i>Terrabacter tumescens</i>	92.4	92.2	92.0	92.8	93.7	91.6	90.6	90.5	90.5	92.7	92.6	91.9	92.7			
<i>Curtobacterium citreum</i>	91.5	91.5	91.1	91.5	92.4	90.9	90.5	89.9	91.9	93.2	93.0	92.0	92.8	92.6		
<i>Clavibacter xyli</i>	91.3	91.4	91.0	91.5	91.9	90.3	90.2	89.8	90.2	92.0	92.0	90.7	90.7	91.8	94.8	
<i>Microbacterium arborescens</i>	90.0	90.0	89.4	90.1	91.2	89.3	89.1	89.0	90.3	91.0	91.5	90.4	90.8	91.3	94.1	93.5

MK-7, but MK-8 is also present in substantial amounts in strain CNRZ 925^T (Table 2). The fatty acids are primarily straight-chain, saturated, iso- and anteiso-methyl, branched fatty acids; 12-methyltetradecanoic (anteiso-C_{15:0}) acid is the predominant fatty acid in strains CNRZ 925^T and CNRZ 926^T (64.3 and 69.1% of the total fatty acids, respectively) (Table 3). Other fatty acids are present at levels of less than 10%. Thus, strains CNRZ 925^T and CNRZ 926^T clearly differ from the previously described brachybacteria, which contain 37.6 to 57.2% 12-methyltetradecanoic (anteiso-C_{15:0}) acid and substantial amounts of 14-methylhexadecanoic (anteiso-C_{17:0}) acid (Table 3).

Our results also demonstrated that teichoic acids with the unusual compound erythritol as the polyol and diaminoglucuronic acid as a substituent occur in strains CNRZ 925^T and CNRZ 926^T (24). In addition to the teichoic acids, galactose (CNRZ 925^T) or galactose and glucose (CNRZ 926^T) were found to constitute separate polysaccharides in the cell walls. An investigation of the cell walls of *B. faecium* DSM 4810^T revealed a similar cell wall structure; erythritol, phosphorus, diaminoglucuronic acid, glucosamine, galactose, and glucose occurred in the cell walls of this organism. On the basis of the results of the cell wall analysis alone we could not determine which constituents belonged to the teichoic acid. Assuming that the cell wall compositions of strains DSM 4810^T, CNRZ 925^T, and CNRZ 926^T are similar, glucose and galactose probably form a separate polysaccharide. Thus, *B. faecium*, the type species of the genus *Brachybacterium* (6), is obviously similar to strains CNRZ 925^T and CNRZ 926^T in cell wall structure. Gvozdyak et al. (11) described the occurrence of rhamnose in *B. nesterenkovi* cells, whereas no rhamnose was present in *B. faecium*. Recently, Takeuchi et al. (29) showed that rhamnose occurs in the cell walls of *B. nesterenkovi*. Our investigation of cell walls, which included extraction of accessory polymers, confirmed this finding and corroborated the hypothesis that

galactose occurs in the cell walls of *Brachybacterium* strains. However, our experiments revealed that glucose is not a cell wall constituent in *B. nesterenkovi* and that 6-deoxytalose is not a cell wall constituent in *B. faecium* (29). Instead (*B. nesterenkovi*) or in addition (*B. faecium*), glucosamine was found. The reason for these differences in the compositions of the carbohydrate moieties of the cell walls is not clear, but the differences might be due to nutritional differences (i.e., addition of brain heart infusion to the culture medium) (29). In this context it seems important to emphasize that when accessory carbohydrate polymers in bacterial cell walls are examined, taxonomically relevant data can be obtained only when polysaccharides and/or teichoic acids are extracted, fractionated, and structurally investigated.

We believe that the chemotaxonomic data described above indicate that strains CNRZ 925^T and CNRZ 926^T belong to the genus *Brachybacterium*. This view was clearly validated by 16S rRNA sequence data. For each of these isolates the levels of sequence similarity to *B. faecium* and *B. conglomeratum*, the closest relatives, were more than 97%, indicating that the organisms are related at the genus level or possibly at the species level. DNA-DNA hybridization experiments performed with *B. faecium* DSM 4810^T, *B. nesterenkovi* DSM 9573^T, *B. conglomeratum* NCIMB 9859, strain CNRZ 925^T, and strain CNRZ 926^T resulted in values well below 70%, the level of relatedness which separates organisms at the species level (27). The levels of DNA relatedness to *B. faecium* DSM 4810^T, *B. nesterenkovi* DSM 9573^T, and *B. conglomeratum* NCIMB 9859 were between 11.4 and 38.1% for both CNRZ 925^T and CNRZ 926^T; these values were similar to the values obtained by Gvozdyak et al. (11) for the relationship between *B. nesterenkovi* and *B. faecium* (22%) and by Takeuchi et al. (29) for the relationships among the five *Brachybacterium* species which they investigated (2 to 49%). The 16S rRNA sequence data and the DNA-DNA hybridization data clearly showed that

FIG. 2. Tree based on the results of a maximum-likelihood analysis of a subset of 16S rRNA sequences from gram-positive bacteria with high DNA G+C contents that were the 40 closest relatives of strains CNRZ 925^T and CNRZ 926^T. The topology of the tree was evaluated by performing distance matrix and maximum-parsimony analyses of more comprehensive data sets containing all available 16S rRNA sequences from gram-positive bacteria with high DNA G+C contents and selected representatives of all known bacterial phyla. Only alignment positions which were occupied by identical residues in at least 50% of all available homologous sequences from gram-positive bacteria with high DNA G+C contents were included in the calculations. The inclusion of more variable positions did not alter the topology of the subtree containing strains CNRZ 925^T and CNRZ 926^T and *Brachybacterium* and *Dermabacter* strains. Multifurcations in the tree are branches for which a relative order could not be determined unambiguously or a common branching order was not significantly supported when different treeing methods were used. Bar = 10% estimated sequence divergence.

CNRZ 925^T and CNRZ 926^T are not members of previously described *Brachybacterium* species. The levels of DNA relatedness between CNRZ 925^T and CNRZ 926^T, corresponding to hybridization values of 10.8 and 17.7% (Table 5), also indicate that these organisms belong to separate species.

Our findings indicate that CNRZ 925^T and CNRZ 926^T represent two new species of the genus *Brachybacterium*. We propose the name *Brachybacterium alimentarium* for CNRZ 925^T and the name *Brachybacterium tyrofermentans* for CNRZ 926^T.

Description of *Brachybacterium alimentarium* sp. nov. *Brachybacterium alimentarium* (a.li.men.ta'ri.um. L. neut. adj. *alimentarium*, pertaining to food) surface colonies are medium sized, vivid lemon colored, low convex, and smooth. Cells vary in shape from coccoid forms in the stationary phase, which occur in small clusters of four to eight cells, to club-shaped rods in the exponential phase, which occur in pairs. Facultatively anaerobic. The optimum temperature is 30°C, and the optimum pH is 7.3. Acid is produced from glucose, but not from

fructose, sucrose, maltose, and lactose. Catalase and oxidase positive. Gelatin is hydrolyzed. Starch, casein, and tributyrin are not hydrolyzed. Tyrosine and xanthine are not utilized. Urease activity is inducible. Nitrate is reduced to nitrite. Tolerance to 14% NaCl.

The cell wall peptidoglycan is based on *meso*-diaminopimelic acid with a D-Asp-D-Glu interpeptide bridge (variation A4_γ). Two teichoic acids are present; one contains erythritol and *N,N'*-diacetyl-2,3-diamino-2,3-dideoxyglucuronic acid, and the other contains *N*-acetylglucosamine and galactose as sugar constituents. The principal menaquinone is MK-7; MK-8 is present in substantial amounts. The long-chain fatty acids are primarily straight-chain, saturated, iso- and anteiso-methyl, branched fatty acids, with 12-methyltetradecanoic (anteiso-C_{15:0}) acid predominating. The G+C content of the DNA is 73 mol% (as determined by the thermal denaturation method). Isolated from the surfaces of Beaufort and Gruyère cheeses. The type strain is CNRZ 925.

Description of *Brachybacterium tyrofermentans* sp. nov.

Brachybacterium tyrofermentans (ty.ro.fer.men'tans. Gr. n. *tyros*, cheese; L. part. adj. *fermentans*, leavening; N. L. adj. *tyrofermentans*, fermenting cheese) surface colonies are medium sized, pale flaxen, low convex, and smooth. Cells vary in shape from coccoid forms in the stationary phase, which occur in large agglomerations, to club-shaped rods in the exponential phase, which occur predominantly in pairs or agglomerations of four cells. Facultatively anaerobic. The optimum temperature is 30°C, and the optimum pH is 7.3. Acid is produced from glucose, but not from fructose, sucrose, maltose, and lactose. Catalase and oxidase positive. Gelatin is hydrolyzed. Starch, casein, and tributyrin are not hydrolyzed. Tyrosine and xanthine are not utilized. Urea is not hydrolyzed. Nitrate and nitrite are reduced. Tolerant to 16% NaCl.

The cell wall peptidoglycan is based on *meso*-diaminopimelic acid with a D-Asp-D-Glu interpeptide bridge (variation A4_γ). One teichoic acid structure is present; this structure contains erythritol, *N*-acetylglucosamine, and *N,N'*-diacetyl-2,3-diamino-2,3-dideoxyglucuronic acid. The principal menaquinone is MK-7. The long-chain fatty acids are primarily straight-chain, saturated, iso- and anteiso-methyl, branched fatty acids, with 12-methyltetradecanoic (anteiso-C_{15:0}) acid predominating. The G+C content of the DNA is 73 mol% (as determined by the thermal denaturation method). Isolated from the surfaces of Beaufort and Gruyère cheeses. The type strain is CNRZ 926.

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TABLE 5. DNA-DNA hybridization data for strains CNRZ 925^T and CNRZ 926^T and other *Brachybacterium* strains

Source of unlabelled DNA	% DNA relatedness with ³⁵ S-labelled DNA from:		
	CNRZ 925 ^T	CNRZ 926 ^T	DSM 20124 ^{Ta}
CNRZ 925 ^T	100	17.7	0.6
CNRZ 926 ^T	10.8	100	
DSM 4810 ^T	19.4	35.8	
DSM 9573 ^T	16.4	11.8	
CCM 2432	11.4	21.5	1.3
NCIMB 9859	38.1	26.2	2.4
CCM 2136	14.3	23.1	0.6
DSM 20124 ^T	2.0		100

^a *A. globiformis* DSM 20124^T was used as a negative control.

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