Taxonomic Study of the Genus *Brachybacterium*: *Brachybacterium nesterenkovi* sp. nov.

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A new species, *Brachybacterium nesterenkovi*, is proposed for a group of coryneform bacteria that were isolated from milk products. These organisms have morphological, biochemical, and chemotaxonomic characteristics that are peculiar to the genus *Brachybacterium*. In contrast to strains of the only previously described species of the genus, *Brachybacterium faecium*, the representatives of the new species lack glycine in their peptidoglycan, although the peptidoglycan is of the same general type, and have large amounts of rhamnose in their cells. The strains of *B. nesterenkovi* exhibit no serological relationship with strains of *B. faecium*; in a numerical phenotypic analysis the two species were easily separated and formed clear-cut clusters. DNA-DNA hybridization between the type strains of *B. faecium* and *B. nesterenkovi* showed a level of homology of 22%. Strain 35 (= IMV Ac-752) is the type strain of *B. nesterenkovi*.

In their investigation of the cell walls of *Arthrobacter* spp. strains NCIB 9859, NCIB 9860^T (T = type strain), and NCIB 9861, “*Micrococcus conglomeratus*” CCM 2134 and CCM 2135, *Micrococcus luteus* CCM 2136 and CCM 2137, and “*Staphylococcus lactis*” CCM 2432, Bogdanovsky et al. (7) determined that the peptidoglycan composition and structure are the same for all of these strains but are not typical of the taxa to which the organisms are assigned. According to the classification of Schleifer and Kandler (29), the peptidoglycan of these bacteria is a peptidoglycan of the meso-diaminopimelic acid (DAP)-D-Glu₂ type (variation A 4γ), which has not been found in any other bacteria. Later, Schleifer and Lang (30) showed that the DNA-DNA hybridization values for these strains are high enough to assume that they belong to one genus. Similarity was also found in their cytochrome patterns. A comparison of the properties of these bacteria with the properties of strains isolated in our laboratory, as well as previously published data, led us to propose a new taxon (“conglomeratus”) for them, presumably at the genus level (23a, 24). Later, the new genus *Brachybacterium* was proposed for some of these bacteria (11), and the members of the “conglomeratus” taxon were shown to be brachybacteria (25). We have found among the brachybacteria isolated in our laboratory a group of strains which differ from the single previously described species of the genus, *Brachybacterium faecium*, in a number of characteristics. The aim of this work was to elucidate the taxonomic position of these bacteria.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Reference strains of *B. faecium* (originally labeled *Arthrobacter* spp. strains NCIB 9859, NCIB 9860^T, and NCIB 9861) were received from the All-Union Collection of Microorganisms (RSFSR), and *M. luteus* CCM 169 was obtained from the Czechoslovak Collection of Microorganisms. Strains IMV Ac-750, IMV Ac-751, IMV Ac-752^T, IMV Ac-753, and IMV Ac-754 were isolated at the Institute of Microbiology and Virology of the Academy of Sciences of the Ukraine in 1984 from milk products by using the methods described previously (16).

Working cultures were grown on beef extract agar (BEA) (18) at 28°C; stock cultures were stored on the same medium at 4°C. To detect the presence of DAP, cells were grown on BEA slants at 34°C for 48 h. To study other chemotaxonomic characteristics, cultures were incubated in 500-ml shake flasks (240 rpm) at 28°C and harvested at the exponential growth phase. For menaquinone pattern determinations cells were grown in a medium containing 10 g of dextrose, 10 g of peptone, 2 g of casein hydrolysate, 2 g of yeast extract, 6 g of NaCl, and 1,000 ml of tap water (pH 7.5). Biomass for other chemical analyses was obtained by using beef extract broth supplemented with 1% glucose and 1% yeast autolysate.

Morphological and biochemical properties. Cell morphology was studied with Gram-stained smears of cultures grown on BEA (18) at 28°C for 14 and 18 to 20 h and 2 to 10 days; colony morphology was studied with cultures grown on BEA, P agar (23), milk agar (6) and glucose-asparagine agar (2) at the same temperature. Motility, spore formation, tolerance to NaCl, and ability to grow at various temperatures were studied by the methods of Labinskaya (18). Tests for carbohydrate and alcohol oxidation and arginine hydrolysis were performed by the methods of Baird-Parker (3). Utilization of sodium salts of organic acids, reduction of nitrate to nitrite, and starch hydrolysis were determined as described by Gordon and Smith (14). Hydrolysis of tyrosine, xanthine, and hypoxanthine and urease formation were studied by the methods of Gordon and Mihm (13). Hydrolysis of Tween 20, Tween 40, Tween 60, and Tween 80 was determined as described by Sierra (31). Esculin hydrolysis was determined by the method of Kvasnikov and Nesterenko (17), and acetoin formation was determined by the method of Baird-Parker (4). Gelatin hydrolysis and growth on Simmons citrate agar were determined by the methods of Collins and Lyne (10). Lecitinase formation was determined by the method of Collins (9). Growth on medium containing 20 µg of furazolidone per ml and the ability to oxidize glycerol in the presence of 0.4 µg of erythromycin per ml were studied as described by Baker (5); the ability to ferment glucose was determined by the method of Schleifer (28).

Susceptibility to antibiotics was determined by the disk method (12), using commercial kits with the following antibiotic concentrations: ampicillin, methicillin, and oxacillin, 10 µg per disk; rifampin, 5 µg per disk; carbenicillin, 25 µg

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per disk; oleandomycin and erythromycin, 15 µg per disk; polymyxin, 300 U per disk; benzylpenicillin, 10 U per disk; and all other antibiotics, 30 µg per disk.

Phenylalanine deaminase, ornithine and lysine decarboxylases, and hydrogen sulfide production were studied by using commercial kits (Indicatory Paper Systems for Complete Identification of Enterobacteria produced by the Enterprize of Bacterial Preparations at the Nizhnij Novgorod Institute of Epidemiology and Microbiology, Nizhnij Novgorod, RSFSR).

Chemotaxonomic characteristics. The presence and form of DAP were determined by ascending paper chromatography (26).

Samples for studying peptidoglycan amino acid composition were prepared by using the method of Schleifer and Kandler (29). The analysis was performed with a model LC-5001 automatic amino acid analyzer (Biotronic, Maintal, Germany), as described previously (27).

Cell monosaccharide patterns were investigated by performing ascending paper chromatography (19).

The presence of mycolic acids was determined as described by Minnikin et al. (22).

Fatty acids were determined as described by Andreev and Galchenko (1), using gas-liquid chromatography.

Menaquinone extraction, purification, and analysis by high-performance thin-layer chromatography and high-performance liquid chromatography on reversed-phase supports were carried out as described previously (15). Menaquinone standards, which were characterized by their mass spectra, were used to identify the extracted menaquinones.

DNA isolation and purification were performed as described by Boháček et al. (8). DNA base composition was determined by using the thermal denaturation method (20) and a model DU-8B (Beckman) spectrophotometer. DNA-DNA hybridization was carried out by using the membrane filter technique (21). Before labeling by in vitro nick translation (type N.5000 nick translation kit [Amersham]) with [α - 32 P]dCTP, DNA from *B. faecium* NCIB 9860^T was treated with restriction endonuclease *Pst*I. Preincubated Hybond C filters (diameter, 5 mm) were loaded with 10 µg of unlabeled single-stranded DNA from *B. faecium* NCIB 9860^T or *Brachybacterium nesterenkovi* IMV Ac-752^T or with *M. luteus* CCM 169^T or sea urchin DNA. The ratios of filter-bound DNA to labeled DNA were about 20:1. The preparations were hybridized for 18 h at 60°C in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 5× Denhardt solution, 0.5% sodium dodecyl sulfate, and 33% formamide. The radioactivity value obtained for *B. faecium* NCIB 9860^T homologous hybrids was defined as 100% homology; the values for heterologous DNA hybrids were normalized to this value.

RESULTS

The five strains which we isolated were characterized by using a number of tests, and these organisms had some common properties (Table 1).

In the stationary growth phase the cultures consisted mainly of coccoid cells which were 0.5 to 1 µm in diameter. When they were transferred to fresh medium after 14 to 20 h, the cells were round, oval, angular, or short rods that were up to 2 µm long. Some cells exhibited V-shaped arrangements.

The cells were gram positive, not acid fast, nonmotile, and nonsporeforming.

Colonies on all of the media which we studied were matte

TABLE 1. Characteristics of *B. nesterenkovi* strains

Characteristic	Strains				
	IMV Ac-750	IMV Ac-751	IMV Ac-752 ^T	IMV Ac-753	IMV Ac-754
Growth in 5% NaCl broth	+	±	—	—	+
Acid produced from ^b :					
Xylose	—	+	±	±	±
Galactose	—	+	+	±	+
Mannose	—	+	—	—	±
Fructose	+	+	±	—	+
Lactose	+	+	+	—	+
Maltose	—	+	+	+	+
D-Melibiose	±	±	+	—	—
Sucrose	—	+	+	±	±
Cellobiose	—	±	+	—	—
Melezitose	—	±	±	—	—
Raffinose and sorbitol	+	—	—	—	—
Starch	—	—	+	+	+
Glycerol	+	+	+	±	±
Utilization of acetate, lactate, pyruvate, and succinate	—	—	+	±	—
NH ₃ produced from arginine after:					
7 days	—	—	—	—	—
21 days	—	+	+	—	—
Hydrolysis of:					
Tyrosine	—	±	—	—	—
Gelatin	—	—	—	—	+
Starch	±	+	+	+	+
Susceptibility to:					
Benzylpenicillin	+	+	+	+	±
Streptomycin	+	+	+	±	+
Neomycin	+	+	±	+	±
Kanamycin	—	+	+	+	+
Polymyxin	—	±	—	—	—

^a +, positive; —, negative; ±, variable.

^b Under aerobic conditions.

and convex and had regular edges, and their surfaces were smooth or rough. They were up to 1.5 mm in diameter (rarely 3 mm on BEA and P agar). Colonies were lemon yellow on BEA and milk agar, somewhat lighter on P agar, and pale greyish white on glucose-asparagine agar.

Cultures grew well on BEA, somewhat less well on milk agar and P agar, and weakly on the glucose-asparagine medium. Cultures grew well in 0 to 0.5% NaCl broth and weakly in 5% NaCl broth, and no growth was observed in medium containing 7.5% NaCl. When the organisms were grown in peptone broth, the medium remained transparent or became translucent, and a flaky sediment was formed. As a rule, a growth ring was not observed.

All of the strains which we studied grew well at 18 to 37°C, fermented glucose, formed acid on glycerol-erythromycin medium, were positive in the test performed with furazolidone, formed acid aerobically from ribose and glucose, hydrolyzed esculin, reduced nitrate to nitrite, and were susceptible to ampicillin, oxacillin, methicillin, carbenicillin, erythromycin, oleandomycin, rifampin, lincomycin, ristomycin, tetracycline, and chloramphenicol. All of the strains were negative for growth on Simmons citrate agar, acid formation from erythrose, arabinose, L-sorbose, rhamnose, trehalose, adonitol, D-arabitol, L-arabitol, and inositol, utilization of oxaloacetate, tartrate, and maleate, phenylalanine deaminase formation, formation of lysine and ornithine decarboxylases, hydrolysis of hypoxanthine, xanthine, urea, Tween 20, Tween 40, Tween 60, and Tween 80, and produc-

TABLE 2. Peptidoglycan amino acids and cell monosaccharide patterns of some *Brachybacterium* strains^a

Strain	Peptidoglycan amino acids								Presence of rhamnose in cells
	Content (μmol/mg)				Molar ratio				
	<i>meso</i> -DAP	Ala	Glu	Gly	<i>meso</i> -DAP	Ala	Glu	Gly	
IMV Ac-750	0.49	0.82	1.4		1	1.7	2.85		+
IMV Ac-751	0.41	0.69	1.3		1	1.7	3.2		+
IMV Ac-752 ^T	0.36	0.66	1.19		1	1.8	3.3		+
IMV Ac-753	0.52	0.9	1.5		1	1.7	2.9		+
IMV Ac-754	0.46	0.82	1.39		1	1.8	3.0		+
NCIB 9859	1.13	2.88	3.72	1.36	1	2.1	3.3	1.2	—
NCIB 9860 ^T	0.30	0.73	1.08	0.39	1	2.3	3.2	1.3	—
NCIB 9861	0.85	1.85	2.68	1.09	1	2.2	3.2	1.3	—

^a Significant amounts of galactose and glucose are present in the cells of all of the strains tested.

tion of lecithinase, hydrogen sulfide, and acetoin. The results of the other biochemical tests are summarized in Table 1.

The principal menaquinones of the strains which we studied were nonhydrogenated menaquinones of the MK-7 type. The peptidoglycan lacked glycine, and the amino acid ratios (Table 2) corresponded to a *meso*-DAP-D-Glu₂ type. The major fatty acids were anteiso-C_{15:0} and anteiso-C_{17:0}. Considerable amounts of galactose, glucose, and rhamnose were found in the cells; mycolic acids were absent. The DNA-DNA homology value for strain IMV Ac-752^T, which was considered typical of the strains studied (25), and *B. faecium* NCIB 9860^T was 22%. There was no homology between *B. faecium* NCIB 9860^T DNA and *M. luteus* CCM 169^T DNA (2%) or between *B. faecium* NCIB 9860^T DNA and sea urchin DNA (0%). The DNA base composition of strain IMV Ac-752^T was 70 mol% G+C.

DISCUSSION

The studies by Bogdanovsky et al. (7) of the murein peptide components of some strains belonging to the genera *Arthrobacter*, *Micrococcus*, and *Staphylococcus* showed that these strains possess a peptidoglycan of the *meso*-DAP-D-Glu₂ type (variation A 4γ). The amino acids in this peptidoglycan are *meso*-DAP, alanine, glutamic acid, and glycine in a ratio of 1:2:3:1 or *meso*-DAP, alanine, and glutamic acid in a ratio of 1:2:3 (in the latter case the peptide subunits lack glycine amide). Collins et al. (11) recently proposed the new genus *Brachybacterium* for several strains that were formerly designated *Arthrobacter* spp. (strains NCIB 9859, NCIB 9860^T, and NCIB 9861) and were studied previously by Bogdanovsky et al. (7). Until now, this genus has been represented by a single species, *B. faecium*, and the genus is characterized by the following chemotaxonomic features: the peptidoglycan is of *meso*-DAP-D-Glu₂ type (variation A 4γ); the principal menaquinone is MK-7; the principal fatty acids are anteiso-C_{15:0} and anteiso-C_{17:0}; mycolic acids are absent; and the G+C content of the DNA is 68 to 72 mol% (as determined by the thermal denaturation method). On the basis of these characteristics, the strains isolated in our laboratory should be assigned to the genus *Brachybacterium*. However, we found some characteristics which distinguish our isolates from *B. faecium* (Table 2).

All of the strains of *B. faecium* have glycine in their peptidoglycan; this compound is absent from the murein of our isolates. The presence of glycine in the peptidoglycan of *B. faecium* strains correlates with the absence of rhamnose in the cells, whereas in the cells of our isolates significant amounts of this carbohydrate are always detected. Our

strains have no serological relationship to representative strains of *B. faecium* (14a). According to the results of a numerical analysis that was based on a considerable number of representatives of the genus *Brachybacterium* and a wide range of characters, the strains of *B. faecium* and our isolates form two separate clusters (25).

The relationship of our isolates to the genus *Brachybacterium*, but not to *B. faecium*, was confirmed by the data on DNA-DNA hybridization between the type strain of *B. faecium* and strain IMV Ac-752^T, which is typical of our isolates. A hybridization value of 22% indicates the relatedness of these organisms at the genus level but not at the subspecies level. This finding agrees with the results obtained by Schleifer and Lang (30) with strain "S. lactis" CCM 2432, similar to our strains, and *Arthrobacter* spp. strains NCIB 9859, NCIB 9860^T, and NCIB 9861, which were assigned to the genus *Brachybacterium*.

On the basis of chemotaxonomic characteristics (Table 3) and DNA homology data, we believe that our isolates should be assigned to a new species of the genus *Brachybacterium*, for which we propose the name *B. nesterenkovi*.

TABLE 3. Differentiation of species of the genus *Brachybacterium*

Characteristic	<i>B. faecium</i> (43 strains) ^a	<i>B. nesterenkovi</i>	
		5 strains	IMV strain Ac-752 ^T
Growth in 7.5% NaCl broth	83 ^b	0 ^b	— ^c
Acid produced from:			
Mannose	95	40	—
Rhamnose	70	0	—
Lactose	28	80	+
Trehalose	77	0	—
Melezitose	81	40	—
Adonitol	83	0	—
D-Arabitol	98	0	—
Gelatin hydrolysis	81	20	—
Susceptibility to:			
Oxacillin	30	100	+
Methicillin	16	100	+
Oleandomycin	20	100	+
Glycine present in peptide subunit of peptidoglycan	100	0	—
Rhamnose present in cells	0	100	+

^a The data for *B. faecium* are for 43 representatives, including strains isolated by us, the type strain, and other reference cultures (25).

^b Percentage of strains positive.

^c +, positive; —, negative.

Description of *Brachybacterium nesterenkovi* sp. nov. *Brachybacterium nesterenkovi* (nes. te. ren. ko' vi.i. N.L. gen. n. *nesterenkovi*, of Nesterenko, named for O. A. Nesterenko, a Ukrainian microbiologist who has made a valuable contribution to the systematics of coryneform and nocardioform bacteria). In the stationary growth phase cells are coccoid (diameter, 0.5 to 1 µm). During the logarithmic growth phase the cells are coccoid, oval, angular, and short rods that vary in size from strain to strain (up to 2 µm long). V-shaped cells are observed.

Colonies are matte and convex with regular edges; their surfaces are usually smooth but may be rough. The colony diameter on BEA is up to 1.5 mm (rarely, 3 mm). The color of the colonies is lemon yellow on BEA and milk agar and pale greyish white on glucose-asparagine agar.

Cultures grow well on BEA and somewhat poorly on milk agar, and growth is weak on glucose-asparagine agar. When cultures are grown in peptone broth, the medium remains transparent or becomes translucent, and some flocculent sediment is formed. A growth ring usually is not observed.

Facultatively anaerobic. Gram positive, not acid fast, nonmotile, and nonsporeforming.

Grows well at 18 to 37°C. Grows on medium containing 20 µg of furazolidone per ml and forms acid from glycerol in the presence of erythromycin. Does not grow on Simmons citrate agar. Does not tolerate NaCl; grows well in the presence of 0 to 0.5% NaCl and weakly in the presence of 5% NaCl and does not grow in the presence of 7.5% NaCl.

Acid is produced aerobically from ribose, galactose, glucose, fructose, maltose, sucrose, and glycerol. Erythrose, arabinose, L-sorbose, rhamnose, trehalose, melezitose, raffinose, adonitol, D-arabitol, L-arabitol, dulcitol, inositol, mannitol, and sorbitol are not attacked. Variable results are observed for acid formation from xylose, mannose, D-melibiose, salicin, and starch. Utilizes tartrate, oxaloacetate, and maleate. Some strains poorly utilize acetate, lactate, pyruvate, and succinate. Phenylalanine deaminase negative and negative for lysine and ornithine decarboxylases. Ammonia production from arginine is detected 21 days after inoculation. Hydrolyzes esculin and starch but not tyrosine, gelatin, xanthine, hypoxanthine, Tween 20, Tween 40, Tween 60, Tween 80, and urea. Lecithinase, hydrogen sulfide, and acetoin negative.

Susceptible to ampicillin, oxacillin, methicillin, carbenicillin, benzylpenicillin, streptomycin, neomycin, kanamycin, erythromycin, oleandomycin, rifampin, lincomycin, ristomycin, tetracycline, and chloramphenicol. Not susceptible to polymyxin.

Peptidoglycan has *meso*-DAP, alanine, and glutamic acid (1:2:3). There are significant amounts of glucose, galactose, and rhamnose in the cells. Mycolic acids are absent. The principal menaquinone is MK-7. The main fatty acids are anteiso-C_{15:0} and anteiso-C_{17:0}. The G+C content of the DNA is 70 mol%.

Isolated from milk and milk products.

The type strain of *B. nesterenkovi* is strain 35 (= IMV Ac-752).

Additional characteristics of the type strain. *B. nesterenkovi* IMV Ac-752^T actively forms acid from D-melibiose, lactose, and starch, weakly oxidizes cellobiose, xylose, and melezitose, and does not attack mannose. Ammonia is formed from arginine within 21 days. Weakly susceptible to neomycin. The G+C content of the DNA is 70 mol% (as determined by the thermal denaturation method). Other characteristics correspond to those described above for the species.

Differential characteristics of *B. nesterenkovi* and *B. faecium* are shown in Table 3.

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