Zimmermannella helvola gen. nov., sp. nov.,
Zimmermannella alba sp. nov., *Zimmermannella*
bifida sp. nov., *Zimmermannella faecalis* sp. nov.
and *Leucobacter albus* sp. nov., novel members
of the family *Microbacteriaceae*

Yi-Chueh Lin,¹ Kazunori Uemori,¹ Dominique A. de Briel,²
Vallapa Arunpaiojana³ and Akira Yokota¹

Correspondence

Yi-Chueh Lin

yilin@genes.nig.ac.jp

¹Laboratory of Bioresources, Institute of Molecular and Cellular Biosciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-0032, Japan

²DADB, Service de Microbiologie, Hôpital Louis-Pasteur, Hôpitaux Civils, 68024 Colmar, France

³Thailand Institute of Scientific and Technological Research (TISTR), 196 Phahonyothin Road, Chatuchak, Bangkok 10900, Thailand

Seven strains of actinobacteria, isolated from soil, wounds, urine, cow faeces, human blood and butter, were characterized by a polyphasic approach to clarify their taxonomic position. On the basis of chemotaxonomy, 16S rRNA gene analysis and DNA relatedness, strain IAM 14851^T can be classified within the cluster of the genus *Leucobacter* and is proposed as a novel species, *Leucobacter albus* sp. nov., with strain IAM 14851^T (= TISTR 1515^T) as the type strain. The other six strains formed a phylogenetically separate branch in the family *Microbacteriaceae*, having the following characteristics: the major menaquinones are MK-8 to MK-10, the DNA G+C content ranges from 62 to 68 mol%, the diamino acid in the cell wall is diaminobutyric acid and the muramic acid in the peptidoglycan is of the acetyl type. The major fatty acids are 12-methyltetradecanoic acid (anteiso-C_{15:0}), hexadecanoic acid (C_{16:0}), 14-methyl-pentadecanoic acid (iso-C_{16:0}) and 14-methyl-hexadecanoic acid (anteiso-C_{17:0}). On the basis of morphological, physiological and chemotaxonomic characteristics, together with DNA–DNA hybridization and 16S rRNA gene sequence comparison, the novel genus *Zimmermannella* gen. nov. is proposed for these six strains. Four novel species are proposed: *Zimmermannella helvola* sp. nov. (type species; type strain IAM 14726^T=NBRC 15775^T=DSM 20419^T=TISTR 1509^T), *Zimmermannella alba* sp. nov. (type strain IAM 14724^T=NBRC 15616^T=TISTR 1510^T), *Zimmermannella bifida* sp. nov. (type strain IAM 14848^T=TISTR 1511^T) and *Zimmermannella faecalis* sp. nov. (type strain IAM 15030^T=NBRC 15706^T=ATCC 13722^T=TISTR 1514^T).

INTRODUCTION

The family *Microbacteriaceae* currently contains 15 genera, which are characterized by B-type peptidoglycan (Schleifer & Kandler, 1972) and unsaturated major menaquinones (Collins & Jones, 1981). At first, the family *Microbacteriaceae* was proposed to accommodate the genera *Agromyces*,

Aureobacterium, *Clavibacter*, *Curtobacterium* and *Microbacterium* (Park *et al.*, 1993). On the basis of 16S rRNA gene sequence data, the family was found to accommodate the genera *Agrococcus* and *Rathayibacter* in addition to the above-mentioned genera (Stackebrandt *et al.*, 1997). The genera *Leucobacter* (Takeuchi *et al.*, 1996) and *Cryobacterium* (Suzuki *et al.*, 1997) have also been shown to represent branches within the family *Microbacteriaceae*. It has been proposed that the genera *Aureobacterium* and *Microbacterium* should be united in the redefined genus *Microbacterium* on the basis of 16S rRNA gene sequence data and chemotaxonomic data (Takeuchi & Hatano, 1998). The new genera *Frigoribacterium* (Kämpfer *et al.*, 2000) and

Published online ahead of print on 16 April 2004 as DOI 10.1099/ijs.0.02741-0.

Abbreviation: DAB, 2,4-diaminobutyric acid.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences determined in this study are given in Fig. 2.

Subtercola (Männistö *et al.*, 2000) have been reported as psychrophilic members of the family. The genera *Leifsonia* (Evtushenko *et al.*, 2000), *Agreia* (Evtushenko *et al.*, 2001), *Mycetocola* (Tsukamoto *et al.*, 2001), *Okibacterium* (Evtushenko *et al.*, 2002) and *Plantibacter* (Behrendt *et al.*, 2002) have also been shown to be members of the family *Microbacteriaceae*.

The aim of the present study was to identify the seven isolates collected from various sources in the culture collection of the Institute of Applied Microbiology, University of Tokyo (Tokyo, Japan). Here we describe the physiological, chemotaxonomic and phylogenetic characteristics of these strains. On the basis of these data, six strains are proposed as constituting a novel genus, *Zimmermannella* gen. nov., containing four new species; the other strain is proposed to be a novel species, *Leucobacter albus* sp. nov.

METHODS

Bacterial strains. Seven strains isolated from various sources and obtained from the above-mentioned culture collection are listed in Table 1. Strains IAM 14848^T and IAM 14851^T were isolated in our laboratory from soil from Thailand. The medium used for isolation was nutrient agar (Difco) containing 20 µg polymyxin B ml⁻¹ and 100 µg cycloheximide ml⁻¹ and was incubated at 27 °C for 7 days. After colonies had formed, the Gram-positive strains were picked up. With the exception of strain IAM 14724^T, the strains were grown in peptone/yeast extract broth supplemented with brain heart infusion (BHI) (Difco), i.e. 1 % peptone, 0.2 % yeast extract, 0.2 % NaCl, 0.2 % D-glucose, 0.2 % BHI, 1.5 % agar (pH 7.0) (PY-BHI medium) and nutrient agar medium containing 0.5 % peptone, 0.3 % meat extract, 0.3 % NaCl and 1.5 % agar (pH 7.0). Strain IAM 14724^T was grown in PY-BHI medium to which 0.1 % MgSO₄·7H₂O had been added. All strains were cultured aerobically at 30 °C.

Morphological, physiological and biochemical characteristics. Cell morphology was determined by using cells grown on PY-BHI agar. Each sample used for scanning electron microscopy with a model S-4500 scanning electron microscope (Hitachi) was prepared by fixing cells with 1 % glutaraldehyde and then dehydrating them

with a graded acetone series and then in a Hitachi model HCP-2 critical point drying apparatus. Motility was determined by the hanging drop method. Unless otherwise indicated, all tests were performed at 25 °C. Catalase activity was determined by bubble formation in a 3 % H₂O₂ solution. Oxidase activity was determined by the oxidation of 1 % tetramethyl-*p*-phenylenediamine on filter paper. Acid production from carbohydrates, assimilation of carbohydrates, nitrate reduction and the hydrolysis of aesculin and gelatin were studied by using API 50 CH and API 20 NE strips (bioMérieux).

Chemical analyses. Cell walls were prepared from approximately 500 mg (dry weight) bacterial cells as described by Schleifer & Kandler (1972). Amino acids in an acid hydrolysate of the cell walls were identified by two-dimensional descending chromatography on cellulose TLC plates (Tokyo Kasei) by the method of Harper & Davis (1979) and by HPLC, as their phenylthiocarbamoyl derivatives, with a model LC-6AD HPLC apparatus (Shimadzu) equipped with a Wakopak WS-PTC column (Wako Pure Chemical Industries, 1989). Analysis of enantiomeric diamino acid isomers was performed according to Sasaki *et al.* (1998). Cell-wall sugars were analysed as described by Mikami & Ishida (1983). Fatty acids were extracted from dried cells, purified and then examined as described previously (Yokota *et al.*, 1993). The glycolate test was performed by using the method of Uchida & Aida (1977).

G+C content and DNA-DNA relatedness. Isolation and purification of chromosomal DNA and estimation of the DNA G+C content were performed by using the methods of Takagi *et al.* (1993). DNA relatedness values were determined as described by Ezaki *et al.* (1989).

16S rRNA gene sequence analyses. The 16S rRNA gene was amplified by a PCR using prokaryotic 16S rRNA gene universal primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1510R (5'-GGCTACCTTGTTACGA-3'). The PCR products were purified using a Sepharose Cl-2B gel (Pharmacia). Sequencing reactions were performed using the ABI PRISM BigDye Terminator cycle sequencing ready reaction kit (Applied Biosystems). The primers used for sequencing were 8F, 704R (5'-TCTACGCATTTCACC-3'), 520F (5'-CAGCAGCCGCCGTAATAC-3'), 1100R (5'-GGGTGCGCTCGT-TG-3'), 926F (5'-AAACTCAAAGGAATTGACGG-3') and 1510R. All PCRs were performed with a Perkin-Elmer Cetus model 9600 thermal cycler. Each extension product resulting from the sequencing reaction was purified through a Centri-Sep spin column (Applied Biosystems) and sequenced by using an ABI model 373S automated DNA sequencer.

Table 1. Strains used in this study

CIP, Collection de l'Institut Pasteur, Paris, France; TISTR, Culture Collection of the Thailand Institute of Scientific and Technological Research, Bangkok, Thailand; ATCC, American Type Culture Collection, Manassas, VA, USA; IFO, Institute for Fermentation, Osaka, Japan.

Strain	Other strain designations	Source of isolation
<i>Z. helvola</i> IAM 14726 ^T	' <i>Brevibacterium helvolum</i> ' (Zimmermann) Lochhead DSM 20419, IFO 15775, TISTR 1509	Butter
<i>Z. alba</i> IAM 14724 ^T	A59836 ^T (D. A. de Briel), IFO 15616 ^T , TISTR 1510 ^T	Urine (Strasbourg University Hospital, France)
<i>Z. bifida</i> IAM 14848 ^T	Isolate S7-7 ^T , TISTR 1511 ^T	Soil of Nakhon Ratchasima Province, Thailand
<i>Z. bifida</i> IAM 15028	CIP 102131, IFO 15777, TISTR 1512	Human blood
<i>Z. bifida</i> IAM 15029	B4657 (D. A. de Briel), IFO 15614, TISTR 1513	Wound (Strasbourg University Hospital, France)
<i>Z. faecalis</i> IAM 15030 ^T	[<i>Corynebacterium bovis</i>] ATCC 13722 ^T , IFO 15706 ^T , TISTR 1514 ^T	Cow faeces
<i>L. albus</i> IAM 14851 ^T	Isolate F-8 ^T , TISTR 1515 ^T	Soil of Uthai Thani Province, Thailand

Phylogenetic analysis. The multiple alignment of sequences, calculation of nucleotide substitution rates (K_{nuc} values; Kimura, 1980), construction of a neighbour-joining phylogenetic tree (Saitou & Nei, 1987) and bootstrap analysis with 1000 replicates for evolution of phylogenetic tree topology (Felsenstein, 1985) were carried out with the CLUSTAL W multiple sequence alignment program (Thompson *et al.*, 1994). The NCBI and DDBJ accession numbers used in this analysis are given in Fig. 2. *Arthrobacter globiformis* ATCC 8010^T (M23411) and *Cellulomonas flavigena* ATCC 482^T (X79463) were used as the outgroup.

RESULTS

Morphological, physiological and biochemical characteristics

All of the strains were Gram-positive, non-sporulating and aerobic. Cells of strain IAM 14726^T usually occurred in pairs with two rings on one side (Fig. 1a). Cells of strains IAM 14726^T, IAM 14724^T, IAM 15030^T and IAM 14851^T were short rods, 0.3–0.4 µm wide and 0.5–1.1 µm long;

Fig. 1. Scanning electron micrographs of cells of *Zimmermannella* strains. (a) *Z. helvola* IAM 14726^T, showing rod-shaped cells with two rings in one side. (b) *Z. bifida* IAM 14848^T, showing branched cells. Bars, 1 µm.

cells of IAM 14848^T, IAM 15028 and IAM 15029 were rods, 0.3–0.4 µm wide and 1.5–3 µm long in PY-BHI medium. Cells of strain IAM 14848^T had a branched shape (Fig. 1b). The cells of all strains formed round, smooth, convex, white colonies. All strains were catalase-positive and negative for nitrate reduction (Table 2). Strains IAM 14848^T, IAM 15028, IAM 15029 and IAM 14724^T were positive for alkaline phosphatase activity. Strains IAM 14726^T and IAM 14848^T produced acid from rhamnose, and strain IAM 15028 produced acid from ribose. Strain IAM 15030^T produced acid from D-glucose, D-fructose, D-mannose, rhamnose, inositol and mannitol. Strain IAM 14851^T produced acid from glycerol, ribose and L-fucose.

Chemical analyses

The strains were divided into four groups according to their major menaquinone compositions (Table 3). Strains IAM 14726^T and IAM 15030^T had MK-9 as the major menaquinone, IAM 14724^T had MK-10, while strains IAM 14848^T, IAM 15028 and IAM 15029 had MK-8 and MK-9. In strain IAM 14851^T, MK-11 was the major menaquinone. The cellular fatty acids of all of the strains mainly comprised anteiso-C_{15:0}, C_{16:0}, iso-C_{16:0} and anteiso-C_{17:0}.

The diamino acid in the peptidoglycan of the strains was 2,4-diaminobutyric acid (DAB) (Table 3). Most of the strains contained L-DAB, except for IAM 15030^T and IAM 14726^T. Strain IAM 15030^T contained homoserine in addition to L-DAB. Strain IAM 14726^T contained L-DAB and D-DAB. The peptidoglycan of strain IAM 14726^T contained glutamate, glycine, alanine and L-DAB in molar ratios of 1.0:1.0:0.8:1.8, corresponding to type B2γ of Schleifer & Kandler (1972). Those of strains IAM 14724^T, IAM 14848^T, IAM 15028 and IAM 15029 contained these amino acids in nearly the same ratios. The peptidoglycan of strain IAM 15030^T contained glutamate, glycine, alanine, L-DAB and homoserine in molar ratios of 1.0:1.9:0.8:0.9:0.2. The peptidoglycan of strain IAM 14851^T contained glutamate, glycine, alanine, L-DAB and γ-aminobutyric acid in molar ratios of 1.0:1.1:1.8:0.8:0.7.

The cell-wall sugars of all the strains are shown in Table 3. The cell-wall sugars of strain IAM 14726^T were rhamnose and 6-deoxytalose, while those of strain IAM 14724^T were rhamnose, 6-deoxytalose and glucose. The cell-wall sugars of strains IAM 14848^T, IAM 15028 and IAM 15029 were rhamnose and glucose, that of strain IAM 15030^T was rhamnose, and those of strain IAM 14851^T were rhamnose, galactose and glucose.

Phylogenetic analysis of the 16S rRNA gene

Nearly complete 16S rRNA gene nucleotide sequences (1480 bp) were determined for the seven strains. The phylogenetic tree constructed using the neighbour-joining method and K_{nuc} values clearly shows that strain IAM 14851^T is in the same cluster as *Leucobacter komagatae* IAM 1093^T, with the other six strains occupying a distinct

Table 2. Phenotypic characteristics of isolates

Strains: 1, *Z. helvola* gen. nov., sp. nov. IAM 14726^T; 2, *Z. alba* sp. nov. IAM 14724^T; 3–5, *Z. bifida* sp. nov. strains IAM 14848^T (3), IAM 15028 (4) and IAM 15029 (5); 6, *Z. faecalis* sp. nov. IAM 15030^T; 7, *L. komagatae* IAM 1093^T; 8, *L. albus* sp. nov. IAM 14851^T.

Characteristic	1	2	3	4	5	6	7	8
Oxidase activity	+	–	–	–	–	+	–	–
Nitrate reduction	–	–	–	–	–	–	–	–
Alkaline phosphatase	–	+	+	+	+	–	–	–
Hydrolysis of gelatin	–	–	–	–	–	–	+	–
Acid production from:								
Glycerol	–	–	–	–	–	–	+	+
Ribose	–	–	–	+	–	–	–	+
D-Glucose	–	–	–	–	–	+	–	–
D-Fructose	–	–	–	–	–	+	–	–
D-Mannose	–	–	–	–	–	+	–	–
Rhamnose	+	–	+	–	–	+	–	–
Inositol	–	–	–	–	–	+	–	–
Mannitol	–	–	–	–	–	+	–	–
L-Fucose	–	–	–	–	–	–	–	+

position in the family *Microbacteriaceae* with 100 % bootstrap confidence (Fig. 2).

The level of 16S rRNA gene sequence similarity between the isolates IAM 14851^T and *L. komagatae* IAM 1093^T was 98.2 %. Strains IAM 14726^T and IAM 14724^T showed 96.1–96.6 % 16S rRNA gene sequence similarity. The 16S

rRNA genes of IAM 14848^T, IAM 15028 and IAM 15029 showed 99.6–99.8 % similarity to each other and 96–98.1 % sequence similarity to the other three strains. The 16S rRNA gene of strain IAM 15030^T showed 98 % sequence similarity to those of strains IAM 14848^T, IAM 15028 and IAM 15029, and 96.3–96.6 % similarity to those of strains IAM 14726^T and IAM 14724^T.

G+C content and DNA–DNA relatedness

The DNA G+C contents were 66 mol% for strain IAM 14851^T, 62 mol% for strains IAM 14848^T, IAM 15028 and IAM 15029, 67 mol% for strains IAM 15030^T and IAM 14726^T and 68 mol% for strain IAM 14724^T (Table 4). The levels of DNA relatedness were analysed, and the results are presented in Table 4. Three strains, IAM 14848^T, IAM 15028 and IAM 15029, were found to have 75–82 % relatedness, while the other four strains showed little DNA relatedness. The DNA–DNA hybridization value between IAM 14851^T and *L. komagatae* IAM 1093^T was 40 % (Table 4).

DISCUSSION

On the basis of phenotypic and genotypic characteristics, we concluded that these strains belong to a novel species or a novel genus in the family *Microbacteriaceae*. Strain IAM 14851^T was assigned to the genus *Leucobacter* on the basis of a 16S rRNA gene sequence comparison. Strain IAM 14851^T and the genus *Leucobacter* share the following chemotaxonomic characteristics: MK-11 is the major menaquinone, branched fatty acids are the major cellular fatty acids and

Table 3. Chemotaxonomic characteristics of isolates

Strains: 1, *Z. helvola* gen. nov., sp. nov. IAM 14726^T; 2, *Z. alba* sp. nov. IAM 14724^T; 3–5, *Z. bifida* sp. nov. strains IAM 14848^T (3), IAM 15028 (4) and IAM 15029 (5); 6, *Z. faecalis* sp. nov. IAM 15030^T; 7, *L. komagatae* IAM 1093^T; 8, *L. albus* sp. nov. IAM 14851^T. Abbreviations: 6-DT, 6-deoxytalose; Gal, galactose; Glc, glucose; Hsr, homoserine; Rha, rhamnose.

Feature	1	2	3	4	5	6	7	8
Menaquinone composition (ratio of peak areas):*								
MK-7	0	14	8	7	5	3	0	0
MK-8	6	10	46	55	60	5	0	0
MK-9	88	16	46	38	35	91	0	13
MK-10	6	60	0	0	0	1	16	0
MK-11	0	6	0	0	0	0	72	77
MK-12	0	0	0	0	0	0	12	10
Cellular fatty acid composition (%):								
16:0	17.4	30.5	17.6	17.6	18.2	10.4	0	5.1
i-16:0	17.9	10.9	17.0	19.3	19.7	27.3	13	23.8
a-15:0	41.6	37.8	49.4	47.9	43.7	42.8	65	49.7
a-17:0	18.9	15.7	13.3	12.1	15.0	15.6	22	16.3
Others	4.2	5.1	2.7	3.1	3.4	3.9	0	5.1
Amino acid(s) in cell wall	L-DAB, D-DAB	L-DAB	L-DAB	L-DAB	L-DAB	Hsr, L-DAB	L-DAB	L-DAB
Cell-wall sugar(s)	Rha, 6-DT	Rha, 6-DT, Glc	Rha, Glc	Rha, Glc	Rha, Glc	Rha	Gal, Glc	Rha, Gal, Glc

*Bold letters indicate predominant components.

Fig. 2. Phylogenetic tree showing the relationships of *Zimmermannella* species within the family *Microbacteriaceae*. The tree is based on a 1440 bp alignment of 16S rRNA gene sequences and was constructed using the neighbour-joining method. Numbers at nodes indicate percentages of bootstrap support derived from 1000 replications.

γ -aminobutyric acid is contained in the B-type peptidoglycan of the cell wall. The G + C content of the DNA of strain IAM 14851^T (66 mol%) is also similar to that of *L. komagatae*. The 40 % level of DNA relatedness for IAM 14851^T and *L. komagatae* IAM 1093^T indicates that they belong to different species. According to Wayne *et al.* (1987), DNA–DNA relatedness of 70 % is considered to be the threshold value for the delineation of genomic species; thus, the 40 % level obtained is low enough to justify the separation of strain IAM 14851^T and *L. komagatae*. The strain can also be differentiated from *L. komagatae* phenotypically (Table 2). Hence, strain IAM 14851^T can be regarded as a novel *Leucobacter* species, for which we propose the name *Leucobacter albus* sp. nov.

The other six strains were collected from urine, wounds,

soil, human blood and an unidentified species from the other culture collections. To clarify the taxonomic positions of these bacteria, their morphology, physiology and chemotaxonomic characteristics, together with DNA–DNA relatedness values and 16S rRNA gene sequence comparisons, were used in the present study. The strains were Gram-positive, aerobic, non-spore-forming, rod-shaped bacteria with a high G + C content (62–68 mol%). They also had the following characteristics: the major menaquinone was MK-8, -9 and/or -10, the diamino acid in the cell wall was DAB and the muramic acid in the peptidoglycan was of the acetyl type. The results of phylogenetic analysis of the 16S rRNA gene sequence revealed that these six strains form a monophyletic and distinct cluster, and that this cluster is independent from any of the subclusters corresponding to established genera within the family *Microbacteriaceae*. On

Table 4. DNA base composition and DNA relatedness of the isolates

Strain	G + C content (mol%)	DNA relatedness (%) with:					
		1	2	3	4	5	6
1. <i>Z. helvola</i> IAM 14726 ^T	67	100	22	13	27	17	23
2. <i>Z. alba</i> IAM 14724 ^T	68	18	100	19	12	21	17
3. <i>Z. bifida</i> IAM 14848 ^T	62	18	17	100	17	12	16
<i>Z. bifida</i> IAM 15028	62	10	14	75	15	14	15
<i>Z. bifida</i> IAM 15029	62	19	20	82	17	16	14
4. <i>Z. faecalis</i> IAM 15030 ^T	67	37	19	15	100	12	13
5. <i>L. komagatae</i> IAM 1093 ^T	66	14	11	19	14	100	40
6. <i>L. albus</i> IAM 14851 ^T	66	13	8	19	15	32	100

Table 5. Differential characteristics of genera of the family *Microbacteriaceae*

Data are from Behrendt *et al.* (2002), Davis *et al.* (1984), Evtushenko *et al.* (2000, 2001, 2002), Groth *et al.* (1996), Kämpfer *et al.* (2000), Männistö *et al.* (2000), Sasaki *et al.* (1998), Takeuchi *et al.* (1996), Takeuchi & Hatano (1998), Tsukamoto *et al.* (2001), Wieser *et al.* (1999) and this study. ND, Not determined.

Genus	Diamino acid*	Acyl type	Major menaquinone(s)	G + C content (mol%)
<i>Zimmermannella</i>	DAB	Acetyl	MK-8–MK-10	62–68
<i>Leucobacter</i>	DAB, GABA	Acetyl	MK-11	66
<i>Microbacterium</i>	Lys/Orn	Glycolyl	MK-11–MK-14	65–72
<i>Okibacterium</i>	Lys	Glycolyl	MK-11, MK-12	67
<i>Plantibacter</i>	DAB	Acetyl	MK-10, MK-11	68–70
<i>Mycetocola</i>	DAB	Acetyl	MK-9, MK-10	64–65
<i>Agrococcus</i>	DAB	Acetyl	MK-11, MK-12	74
<i>Cryobacterium</i>	DAB	ND	MK-10	65
<i>Agromyces</i>	DAB	Acetyl	MK-12	70–76
<i>Leifsonia</i>	DAB	ND	MK-10, MK-11	66–73
<i>Rathayibacter</i>	DAB	ND	MK-10	63–72
<i>Frigoribacterium</i>	Lys	Acetyl	MK-9	71·7
<i>Subtercola</i>	DAB	Acetyl	MK-9, MK-10	64–68
<i>Agreia</i>	DAB	ND	MK-10	67
<i>Clavibacter</i>	DAB	Acetyl	MK-9, MK-10	67–78
<i>Curtobacterium</i>	Orn	Acetyl	MK-9	68–75

*GABA, γ -Aminobutyric acid; Lys, lysine; Orn, ornithine.

the basis of the above data, these seven strains should belong to a novel genus in the family *Microbacteriaceae*. Hence, we propose a novel genus, *Zimmermannella* gen. nov. The characteristics used for differentiation at the genus level between the novel genus and other genera of the *Microbacteriaceae* are shown in Table 5. In the family *Microbacteriaceae*, most genera have DAB as a diamino acid and acetyl-type muramic acid in the peptidoglycan. However, the genus *Zimmermannella* can be distinguished from the other genera by the major menaquinone and the DNA G + C content. No genera of the *Microbacteriaceae* have shown MK-8 to MK-10 as the major menaquinones and G + C contents from 62 to 68 mol%, except the genus *Zimmermannella*.

The six strains in the genus *Zimmermannella* can be divided into four novel species according to the results of our DNA relatedness test (Table 4). These species can be distinguished from one another on the basis of both their morphological/biochemical characteristics and DNA–DNA relatedness values. We therefore propose the following classification: strains IAM 14726^T and IAM 15030^T are the type strains of *Zimmermannella helvola* sp. nov. and *Zimmermannella faecalis* sp. nov., respectively, strain IAM 14724^T is the type strain of *Zimmermannella alba* sp. nov. and strains IAM 14848^T, IAM 15028 and IAM 15029 are members of *Zimmermannella bifida* sp. nov. Differential characteristics for these four species are summarized in Table 3.

Description of *Zimmermannella* gen. nov.

Zimmermannella (Zim.mer.man'nel.la. N.L. fem. n. *Zimmermannella* named after O. E. R. Zimmermann, a German microbiologist, who first recognized the species '*Brevibacterium helvolum*').

Cells are Gram-positive, aerobic, non-motile, short rods or rods. Endospores are not produced. Colonies are circular, convex, smooth and generally white on PY–BHI agar. The optimal temperature for growth is generally 30 °C. Catalase is always produced, while oxidase is only sometimes produced. Nitrate reduction, β -galactosidase, α -glucosidase and aesculin hydrolysis are not found. The cell-wall peptidoglycan contains DAB as a diamino acid, the major cell-wall sugar is rhamnose and the muramic acid of the cell wall is of the acetyl type. The major isoprenoid quinones are MK-8 to MK-10. The major fatty acids are C_{16:0}, iso-C_{16:0}, anteiso-C_{15:0} and anteiso-C_{17:0}. The G + C content of the DNA is 62–68 mol%. Forms an independent phylogenetic cluster in the family *Microbacteriaceae*. The observed samples have been isolated from human wounds, urine, cow faeces, butter, human blood and soil. The type species is *Zimmermannella helvola*.

Description of *Zimmermannella helvola* sp. nov.

Zimmermannella helvola (hel'vo.la. L. fem. adj. *helvola* pale yellow).

Displays the following properties in addition to those given in the genus description. Cells are $0.3\text{--}0.5 \times 0.8\text{--}1.1\text{ }\mu\text{m}$ and usually occur in pairs. Two rings are formed on one side of the cells. Oxidase is produced. Acid is produced from rhamnose. The following tests are negative: reduction of nitrite, nitrate respiration, liquefaction of gelatin, urease, alkaline phosphatase and acid production from glycerol, ribose, D-glucose, D-fructose, D-mannose, inositol, mannitol and D-fucose. The cell-wall peptidoglycan contains L- and D-DAB as diamino acids. The major isoprenoid quinone is MK-9. The G + C content of the DNA is 67 mol%.

The type strain, IAM 14726^T (=NBRC 15775^T=DSM 20419^T=TISTR 1509^T), was isolated from butter.

Description of *Zimmermannella alba* sp. nov.

Zimmermannella alba (al'ba. L. fem. adj. *alba* white).

Displays the following properties in addition to those given in the genus description. Cells are $0.3\text{--}0.4 \times 0.5\text{--}1.1\text{ }\mu\text{m}$. Oxidase is not produced. Alkaline phosphatase is found. The following tests are negative: reduction of nitrite, nitrate respiration, liquefaction of gelatin, urease and acid production from glycerol, ribose, D-glucose, D-fructose, D-mannose, rhamnose, inositol, mannitol and L-fucose. The cell-wall peptidoglycan contains L-DAB as a diamino acid. The major isoprenoid quinone is MK-10. The G + C content of the DNA is 68 mol%.

The type strain, IAM 14724^T (=NBRC 15616^T=TISTR 1510^T), was isolated from human urine.

Description of *Zimmermannella bifida* sp. nov.

Zimmermannella bifida (bi'fi.da. L. fem. adj. *bifida* divided into two parts).

Displays the following properties in addition to those given in the genus description. Cells are $0.3\text{--}0.4 \times 1.5\text{--}3.0\text{ }\mu\text{m}$. Branched shapes are formed. Oxidase is not produced. Alkaline phosphatase is produced. The following tests are negative for all strains: reduction of nitrite, nitrate respiration, liquefaction of gelatin, urease and acid production from glycerol, D-glucose, D-fructose, D-mannose, inositol, mannitol and L-fucose. The cell-wall peptidoglycan contains L-DAB as a diamino acid. The major isoprenoid quinones are MK-8 and MK-9. The G + C content of the DNA is 62 mol%.

The samples observed were isolated from human wounds, human blood and soil. The type strain is IAM 14848^T (=TISTR 1511^T).

Description of *Zimmermannella faecalis* sp. nov.

Zimmermannella faecalis (fae.ca'lis. N.L. fem. adj. *faecalis* faecal).

Displays the following properties in addition to those

given in the genus description. Cells are $0.3\text{--}0.4 \times 0.5\text{--}1.1\text{ }\mu\text{m}$. Oxidase is produced. Acid is produced from D-glucose, D-fructose, D-mannose, rhamnose, inositol and mannitol. The following tests are negative: reduction of nitrite, nitrate respiration, liquefaction of gelatin, urease, alkaline phosphatase and acid production from glycerol, ribose and L-fucose. The cell-wall peptidoglycan contains L-DAB as a diamino acid. The major isoprenoid quinone is MK-9. The G + C content of the DNA is 67 mol%.

The type strain, IAM 15030^T (=NBRC 15706^T=ATCC 13722^T=TISTR 1514^T), was isolated from cow faeces.

Description of *Leucobacter albus* sp. nov.

Leucobacter albus (al'bus. L. masc. adj. *albus* white).

Cells are Gram-positive, aerobic rods, $0.3\text{--}0.4 \times 0.5\text{--}1.1\text{ }\mu\text{m}$, non-sporulating and non-motile. Smooth, white colonies are produced on PY-BHI agar. The optimal temperature for growth is 30 °C. Catalase is produced, but oxidase is not. Acid is produced from glycerol, ribose and L-fucose. The following tests are negative: reduction of nitrate or nitrite, nitrate respiration, liquefaction of gelatin, hydrolysis of aesculin, urease, alkaline phosphatase, β -galactosidase and α -glucosidase and acid production from D-glucose, D-fructose, D-mannose, rhamnose, inositol and mannitol. The cell-wall peptidoglycan contains L-DAB and γ -aminobutyric acid; the muramic acid of the cell wall is of the acetyl type. The major isoprenoid quinone is MK-11. The major fatty acids are anteiso-C_{15:0}, anteiso-C_{17:0} and iso-C_{16:0}. The G + C content of the DNA is 66 mol%.

The type strain, IAM 14851^T (=TISTR 1515^T), was isolated from soil.

NOTE ADDED IN PROOF

After this paper was accepted for publication, the description of *Pseudoclavibacter helvolus* for '*Brevibacterium helvolum*' DSM 20419 was published (Manaia *et al.*, 2004).

ACKNOWLEDGEMENTS

The authors would like to thank Dr K. Suzuki for enantiomeric analysis of cell-wall amino acids. We are grateful to Professor H. G. Trüper (University of Bonn, Bonn, Germany) for his help in the latinization of the new genus and species names.

REFERENCES

- Behrendt, U., Ulrich, A., Schumann, P., Naumann, D. & Suzuki, K. (2002). Diversity of grass-associated *Microbacteriaceae* isolated from the phyllosphere and litter layer after mulching the sward; polyphasic characterization of *Subtercola pratensis* sp. nov., *Curtobacterium herbarum* sp. nov. and *Plantibacter flavus* gen. nov., sp. nov. *Int J Syst Evol Microbiol* 52, 1441–1454.

- Collins, M. D. & Jones, D. (1981). The distribution of isoprenoid quinone structural types in bacteria and their taxonomic implications. *Microbiol Rev* **45**, 316–354.
- Davis, M. J., Gillaspsie, A. G., Jr, Vidaver, A. K. & Harris, R. W. (1984). *Clavibacter*: a new genus containing some phytopathogenic coryneform bacteria, including *Clavibacter xyli* subsp. *xyli* sp. nov., subsp. nov. and *Clavibacter xyli* subsp. *cynodontis* subsp. nov., pathogens that cause ratoon stunting disease of sugarcane and Bermudagrass stunting disease. *Int J Syst Bacteriol* **34**, 107–117.
- Evtushenko, L. I., Dorofeeva, L. V., Subbotin, S. A., Cole, J. R. & Tiedje, J. M. (2000). *Leifsonia poae* gen. nov., sp. nov., isolated from nematode galls on *Poa annua*, and reclassification of '*Corynebacterium aquaticum*' Leifson 1962 as *Leifsonia aquatica* (ex Leifson 1962) gen. nov., nom. rev., comb. nov. and *Clavibacter xyli* Davis et al. 1984 with two subspecies as *Leifsonia xyli* (Davis et al. 1984) gen. nov., comb. nov. *Int J Syst Evol Microbiol* **50**, 371–380.
- Evtushenko, L. I., Dorofeeva, L. V., Dobrovolskaya, T. G., Streshinskaya, G. M., Subbotin, S. A. & Tiedje, J. M. (2001). *Agreia bicolorata* gen. nov., sp. nov., to accommodate actinobacteria isolated from narrow reed grass infected by the nematode *Heteroanguina graminophila*. *Int J Syst Evol Microbiol* **51**, 2073–2079.
- Evtushenko, L. I., Dorofeeva, L. V., Krausova, V. I., Gavrish, E. Y., Yashina, S. G. & Takeuchi, M. (2002). *Okibacterium fritillariae* gen. nov., sp. nov., a novel genus of the family Microbacteriaceae. *Int J Syst Evol Microbiol* **52**, 987–993.
- Ezaki, T., Hashimoto, Y. & Yabuuchi, E. (1989). Fluorometric deoxyribonucleic acid-deoxyribonucleic acid hybridization in micro-dilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. *Int J Syst Bacteriol* **39**, 224–229.
- Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.
- Groth, I., Schumann, P., Weiss, N., Martin, K. & Rainey, F. A. (1996). *Agrococcus jenensis* gen. nov., sp. nov., a new genus of actinomycetes with diaminobutyric acid in the cell wall. *Int J Syst Bacteriol* **46**, 234–239.
- Harper, J. J. & Davis, G. H. G. (1979). Two-dimensional thin-layer chromatography for amino acid analysis of bacterial cell walls. *Int J Syst Bacteriol* **29**, 56–58.
- Kämpfer, P., Rainey, F. A., Andersson, M. A., Nurmiäho Lassila, E.-L., Ulrych, U., Busse, H.-J., Weiss, N., Mikkola, R. & Salkinoja-Salonen, M. (2000). *Frigoribacterium faeni* gen. nov., sp. nov., a novel psychrophilic genus of the family Microbacteriaceae. *Int J Syst Evol Microbiol* **50**, 355–363.
- Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* **16**, 111–120.
- Manaia, C. M., Nogales, B., Weiss, N. & Nunes, O. C. (2004). *Gulosibacter molinivorax* gen. nov., sp. nov., a molinate-degrading bacterium, and classification of '*Brevibacterium helvolum*' DSM 20419 as *Pseudoclavibacter helvolus* gen. nov., sp. nov. *Int J Syst Evol Microbiol* **54**, 783–789.
- Männistö, M. K., Schumann, P., Rainey, F. A., Kämpfer, P., Tsitko, I., Tirola, M. A. & Salkinoja-Salonen, M. S. (2000). *Subtercola boreus* gen. nov., sp. nov. and *Subtercola frigoramans* sp. nov., two new psychrophilic actinobacteria isolated from boreal groundwater. *Int J Syst Evol Microbiol* **50**, 1731–1739.
- Mikami, H. & Ishida, Y. (1983). Post-column fluorometric detection of reducing sugars in high-performance liquid chromatography using arginine. *Bunseki Kagaku* **32**, E207–E210.
- Park, Y. H., Suzuki, K., Yim, D. G. & 7 other authors (1993). Suprageneric classification of peptidoglycan group B actinomycetes by nucleotide sequencing of 5S ribosomal RNA. *Antonie van Leeuwenhoek* **64**, 307–313.
- Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Sasaki, J., Chijimatsu, M. & Suzuki, K.-I. (1998). Taxonomic significance of 2,4-diaminobutyric acid isomers in the cell wall peptidoglycan of actinomycetes and reclassification of *Clavibacter toxicus* as *Rathayibacter toxicus* comb. nov. *Int J Syst Bacteriol* **48**, 403–410.
- Schleifer, K. H. & Kandler, O. (1972). Peptidoglycan of bacterial cell walls and their taxonomic implications. *Bacteriol Rev* **36**, 407–477.
- Stackebrandt, E., Rainey, F. & Ward-Rainey, N. L. (1997). Proposal for a new hierarchic classification system, *Actinobacteria* classis nov. *Int J Syst Bacteriol* **47**, 479–491.
- Suzuki, K., Sasaki, J., Uramoto, M., Nakase, T. & Komagata, K. (1997). *Cryobacterium psychrophilum* gen. nov., sp. nov., nom. rev., comb. nov., an obligately psychrophilic actinomycete to accommodate '*Curtobacterium psychrophilum*' Inoue and Komagata 1976. *Int J Syst Bacteriol* **47**, 474–478.
- Tagaki, H., Shida, O., Kadowaki, K., Komagata, K. & Udaka, S. (1993). Characterization of *Bacillus brevis* with descriptions of *Bacillus migulanus* sp. nov., *Bacillus choshinensis* sp. nov., *Bacillus parabrevis* sp. nov., and *Bacillus galactophilus* sp. nov. *Int J Syst Bacteriol* **43**, 221–231.
- Takeuchi, M. & Hatano, K. (1998). Union of the genera *Microbacterium* Orla-Jensen and *Aureobacterium* Collins et al. in a redefined genus *Microbacterium*. *Int J Syst Bacteriol* **48**, 739–747.
- Takeuchi, M., Weiss, N., Schumann, P. & Yokota, A. (1996). *Leucobacter komagatae* gen. nov., sp. nov., a new aerobic Gram-positive, nonsporulating rod with 2,4-diaminobutyric acid in the cell wall. *Int J Syst Bacteriol* **46**, 967–971.
- Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.
- Tsukamoto, T., Takeuchi, M., Shida, O., Murata, H. & Shirata, A. (2001). Proposal of *Mycetocola* gen. nov. in the family Microbacteriaceae and three new species, *Mycetocola saprophilus* sp. nov., *Mycetocola tolaasinivorans* sp. nov. and *Mycetocola lacteus* sp. nov., isolated from cultivated mushroom, *Pleurotus ostreatus*. *Int J Syst Evol Microbiol* **51**, 937–944.
- Uchida, K. & Aida, A. (1977). Acyl type of bacterial cell wall: its simple identification by colorimetric method. *J Gen Appl Microbiol* **23**, 249–260.
- Wako Pure Chemical Industries (1989). *Technical Note on the System of PTC-amino Acid Analysis*. Osaka: Wako Pure Chemical Industries (in Japanese).
- Wayne, L. G., Brenner, D. J., Colwell, R. R. & 9 other authors (1987). International Committee on Systematic Bacteriology. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37**, 463–464.
- Wieser, M., Schumann, P., Martin, K., Altenburger, P., Burghardt, J., Lubitz, W. & Busse, H.-J. (1999). *Agrococcus citreus* sp. nov., isolated from a medieval wall painting of the chapel of castle Herberstein (Austria). *Int J Syst Bacteriol* **49**, 1165–1170.
- Yokota, A., Takeuchi, M., Sakane, T. & Weiss, N. (1993). Proposal of six new species in the genus *Aureobacterium* and transfer of *Flavobacterium esteraromaticum* Omelianski to the genus *Aureobacterium* as *Aureobacterium esteraromaticum* comb. nov. *Int J Syst Bacteriol* **43**, 555–564.