Yonghaparkia alkaliphila gen. nov., sp. nov., a novel member of the family *Microbacteriaceae* isolated from an alkaline soil

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Two Gram-positive, non-motile, non-spore-forming and rod-shaped actinomycete strains, KSL-113^T and KSL-133, were isolated from an alkaline soil in Korea, and their taxonomic positions were investigated by using a polyphasic approach. The strains grew optimally at 30 °C and pH 9.0. Phenotypic, phylogenetic and genetic similarities indicated that strains KSL-113^T and KSL-133 represent the same species. Phylogenetic analyses based on 16S rRNA gene sequences showed that strains KSL-113^T and KSL-133 fell within the family *Microbacteriaceae* of the suborder *Micrococcineae*, the highest 16S rRNA gene sequence similarity values (98.2 %) being obtained with respect to *Microcella putealis* CV-2^T. The 16S rRNA gene sequence similarities between strains KSL-113^T and KSL-133 and the other members of the family *Microbacteriaceae* used in the phylogenetic analysis were less than 96.0 %. Strains KSL-113^T and KSL-133 could be clearly distinguished from members of the family *Microbacteriaceae* on the basis of differences in chemotaxonomic properties, including the predominant menaquinone type, the cell-wall peptidoglycan type and the fatty acid profile. Accordingly, on the basis of the combined phenotypic, chemotaxonomic and phylogenetic data, strains KSL-113^T and KSL-133 constitute a novel genus and species of the family *Microbacteriaceae*, for which the name *Yonghaparkia alkaliphila* gen. nov., sp. nov. is proposed. The type strain of *Yonghaparkia alkaliphila* is KSL-113^T (= KCTC 19126^T = CIP 108920^T).

The family *Microbacteriaceae* was proposed by Park *et al.* (1993) to accommodate the Gram-positive actinomycete genera *Agromyces*, *Aureobacterium*, *Clavibacter*, *Curto-bacterium* and *Microbacterium*, all of which contain peptidoglycan of type B. Stackebrandt *et al.* (1997) assigned two more genera, *Rathayibacter* (Zgurskaya *et al.*, 1993) and *Agrococcus* (Groth *et al.*, 1996), to the family *Microbacteriaceae*. Subsequently, 14 more genera – *Leucobacter* (Takeuchi *et al.*, 1996), *Cryobacterium* (Suzuki *et al.*, 1997), *Frigoribacterium* (Kämpfer *et al.*, 2000), *Leifsonia* (Evtushenko *et al.*, 2000), *Subtercola* (Männistö *et al.*, 2000), *Mycetocola* (Tsukamoto *et al.*, 2001), *Agreia* (Evtushenko *et al.*, 2001), *Okibacterium* (Evtushenko *et al.*, 2002), *Plantibacter* (Behrendt *et al.*, 2002), *Rhodoglobus* (Sheridan *et al.*, 2003), *Salinibacterium* (Han *et al.*, 2003), *Gulosibacter*

and *Pseudoclavibacter* (Manaia *et al.*, 2004) and *Microcella* (Tiago *et al.*, 2005) – have been assigned to the family, and the genera *Microbacterium* and *Aureobacterium* were unified (Takeuchi & Hatano, 1998). The genus *Zimmermannella* was proposed by Lin *et al.* (2004) as a member of the family *Microbacteriaceae*, but the type species of the genus *Zimmermannella helvola* is a later homotypic synonym of *Pseudoclavibacter helvolus* and the genus *Zimmermannella* is therefore illegitimate. In this study, we describe two Gram-positive and alkaliphilic bacterial strains, KSL-113^T and KSL-133, which were found to be most closely related phylogenetically to the family *Microbacteriaceae* on the basis of 16S rRNA gene sequence comparisons. To our knowledge, no alkaliphilic micro-organism has been assigned to the family *Microbacteriaceae*. Accordingly, the aim of the present study was to determine the exact taxonomic positions of strains KSL-113^T and KSL-133 by using a polyphasic approach.

An alkaline soil sample collected from Kwangchun, Korea, was used as the source for the isolation of bacterial strains. Strains KSL-113^T and KSL-133 were isolated by means of the standard dilution plating technique, at 30 °C on

Abbreviation: DAB, 2,4-diaminobutyric acid.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains KSL-113^T and KSL-133 are DQ256087 and DQ256088, respectively.

The cellular fatty acid compositions of strains KSL-113^T and KSL-133 are presented in a supplementary table available in IJSEM Online.

10 × diluted nutrient agar (NA; Difco) with the pH adjusted to 10.0. The morphological, physiological and biochemical characteristics of strains KSL-113^T and KSL-133 were investigated using routine cultivation at 30 °C on 2 × diluted NA with the pH adjusted to 9.0. Cell morphology was examined by using light microscopy (E600; Nikon) and transmission electron microscopy. Flagellation was determined by using a Philips CM-20 transmission electron microscope with cells from exponentially growing cultures: for this purpose, the cells were negatively stained with 1% (w/v) phosphotungstic acid and the grids were examined after being air-dried. The Gram reaction was determined by using the bioMérieux Gram stain kit according to the manufacturer's instructions. Growth at various temperatures (4–45 °C) was measured on 2 × diluted NA (pH 9.0). Growth in the absence of NaCl and at various NaCl concentrations (0.5 and 1.0–5.0%, w/v, at intervals of 1.0%) was investigated in 2 × diluted trypticase soy broth prepared according to the formula of the Difco medium except that no NaCl was used. The pH range for growth was determined in 2 × diluted nutrient broth (Difco) adjusted, prior to sterilization, to various pH values (pH 4.5–10.5, at intervals of 0.5 pH units) by the addition of HCl or Na₂CO₃. Growth under anaerobic conditions was determined after incubation in an anaerobic chamber on 2 × diluted NA (pH 9.0) and on 2 × diluted NA (pH 9.0) supplemented with nitrate, both of which had been prepared anaerobically using nitrogen. Catalase and oxidase activities and the hydrolysis of casein, gelatin, hypoxanthine, starch, Tweens 20, 40, 60 and 80, tyrosine, urea and xanthine were determined as described by Cowan & Steel (1965), but with the modification that the pH of the media was adjusted to 9.0. Aesculin hydrolysis and nitrate reduction were studied as described previously (Lanyi, 1987), with the modification that the pH of the media was adjusted to 9.0. The utilization of substrates as sole carbon and energy sources was tested according to the method of Kämpfer *et al.* (1991), with the modification that the pH of the media was adjusted to 9.0. Antibiotic sensitivity was tested on plates with 2 × diluted NA (pH 9.0) and antibiotic discs containing the following antibiotics: polymyxin B (100 U), streptomycin (50 µg), penicillin G (20 U), chloramphenicol (100 µg), ampicillin (10 µg), cephalothin (30 µg), gentamicin (30 µg), novobiocin (5 µg), tetracycline (30 µg), kanamycin (30 µg), lincomycin (15 µg), oleandomycin (15 µg), neomycin (30 µg) and carbenicillin (100 µg). Enzyme activity was tested by using the API ZYM system (bioMérieux); the cell suspension used to inoculate the system was prepared using phosphate buffer (pH 9.0).

Cell biomass for DNA extraction and for analyses of the cell wall, isoprenoid quinones and polar lipids was obtained from cultures grown in 2 × diluted nutrient broth at 30 °C. Chromosomal DNA was isolated and purified according to the method described previously (Yoon *et al.*, 1996), with the exception that RNase T1 was used in combination with RNase A to minimize contamination with RNA. The 16S rRNA gene sequence was amplified by using a PCR with two

universal primers as described previously (Yoon *et al.*, 1998). Sequencing of the amplified 16S rRNA gene and phylogenetic analysis were performed as described by Yoon *et al.* (2004). The DNA G+C content was determined according to the method of Tamaoka & Komagata (1984), with the modification that the DNA was hydrolysed and the resultant nucleotides were analysed by reversed-phase HPLC. The presence or absence of diaminopimelic acid in the peptidoglycan was determined according to the method described by Komagata & Suzuki (1987). Preparation of the cell walls and determination of the peptidoglycan structure were carried out by using modified methods of Schleifer & Kandler (1972) and MacKenzie (1987). Isoprenoid quinones were extracted according to the method of Komagata & Suzuki (1987) and analysed using reversed-phase HPLC and a YMC ODS-A (250 × 4.6 mm) column. Polar lipids were extracted according to the procedures described by Minnikin *et al.* (1984) and were identified by two-dimensional TLC followed by spraying with the appropriate detection reagents (Minnikin *et al.*, 1984; Komagata & Suzuki, 1987). For analysis of fatty acid methyl esters, cell mass of strains KSL-113^T and KSL-133 was harvested from plates with 2 × diluted NA (pH 9.0) after incubation for 10 days at 30 °C. Fatty acid methyl esters were extracted and prepared according to the standard protocol of the MIDI/Hewlett Packard Microbial Identification System (Sasser, 1990).

The cells of strains KSL-113^T and KSL-133 were Gram-positive and non-spore-forming short rods or rods. Strains KSL-113^T and KSL-133 showed optimal growth at pH 9.0; growth occurred at pH 7.5, but not at pH 7.0. The two strains were identical in all of the morphological, cultural, physiological and biochemical characteristics tested in this study, except with regard to the utilization of some substrates. The phenotypic properties of strains KSL-113^T and KSL-133 are given in the genus and species descriptions (see below) or are shown in Table 1. The almost-complete 16S rRNA gene sequences of strains KSL-113^T and KSL-133 were identical and comprised 1476 nt, representing approximately 96% of the *Escherichia coli* 16S rRNA gene sequence. Comparative 16S rRNA gene sequence analyses revealed that strains KSL-113^T and KSL-133 were phylogenetically most closely affiliated to the family *Microbacteriaceae* of the suborder *Micrococcineae*, particularly with respect to *Microcella putealis* CV-2^T and *Frigoribacterium faeni* DSM 10309^T (Fig. 1). In the neighbour-joining tree based on 16S rRNA gene sequences, strains KSL-113^T and KSL-133 joined the phylogenetic lineage of *Microcella putealis* CV-2^T at a bootstrap resampling value of 100% (Fig. 1). The relationship between strains KSL-113^T and KSL-133 and *Microcella putealis* CV-2^T was maintained in trees generated with the maximum-likelihood and maximum-parsimony algorithms (Fig. 1). The similarity values between the 16S rRNA gene sequences of strains KSL-113^T and KSL-133 and those of *Microcella putealis* CV-2^T and *Frigoribacterium faeni* DSM 10309^T were 98.2 and 96.7%, respectively. Strains KSL-113^T

Table 1. Differential phenotypic characteristics of strains KSL-113^T and KSL-133 (*Yonghaparkia* gen. nov.) and the genera *Microcella*, *Frigoribacterium* and *Mycetocola*

Data for reference genera were taken from Tiago *et al.* (2005) (*Microcella*), Kämpfer *et al.* (2000) (*Frigoribacterium*) and Tsukamoto *et al.* (2001) (*Mycetocola*). +, Positive; –, negative; V, variable (results for the type strain of the type species in parentheses); ND, no data available. All taxa are positive for the Gram stain and catalase activity.

Characteristic	<i>Yonghaparkia</i>	<i>Microcella</i>	<i>Frigoribacterium</i>	<i>Mycetocola</i>
Cell size (µm)	0.2–0.4 × 0.6–1.4	0.4 × 0.8–1.6	0.2–0.3 × 1.0–1.5	0.2–0.4 × 2.0–3.5
Motility	–	ND	+	–
Optimal temperature for growth (°C)	30	35	4–10	25
Oxidase activity	+	–	–	–
Growth at:				
4 °C	–	–	+	–
30 °C	+	+	–	+
Hydrolysis of:				
Aesculin	+	–	+	+
Casein	+	+	ND	–
Gelatin	–	–	ND	–
Utilization of:				
D-Glucose	–	+	+	ND
D-Fructose	–	+	+	ND
D-Galactose	V (+)	V (+)	+	ND
D-Cellobiose	–	+	+	ND
D-Mannose	–	+	+	ND
D-Trehalose	–	V (–)	+	ND
D-Xylose	–	V (+)	+	ND
L-Arabinose	–	+	+	ND
Sucrose	–	+	+	ND
Maltose	–	+	+	ND
Citrate	–	ND	V (+)	ND
Pyruvate	+	ND	–	ND
Diamino acid	DAB	D-Lys	D-Lys	D-Lys
Predominant menaquinone(s)	MK-12	MK-12, MK-13	MK-9	MK-10
Major fatty acids (> 10% of total fatty acids)	anteiso-C _{15:0} , iso-C _{16:0} , anteiso-C _{17:0}	iso-C _{16:0} , anteiso-C _{15:0} , iso-C _{14:0} , iso-C _{15:0}	anteiso-C _{15:0} , iso-C _{16:0} , anteiso-C _{15:0} -DMA*	anteiso-C _{15:0} , anteiso-C _{17:0}
DNA G + C content (mol%)	71.1–71.6	68.8–70.7	71	63.9–65.2

*anteiso-C_{15:0}-DMA, 1,1-Dimethyl-anteiso-pentadecane.

Fig. 1. Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the positions of strain KSL-113^T and related taxa. Bootstrap values (from 1000 replications) are shown as percentages at each node; only values of 50% or more are shown. Dots indicate that the corresponding nodes were also recovered in trees generated with the maximum-likelihood and maximum-parsimony algorithms. *Cellulomonas flavigena* DSM 20109^T was used as an outgroup (GenBank accession no. X83799; not shown). Bar, 0.01 substitutions per nucleotide position.

and KSL-133 exhibited 16S rRNA gene sequence similarity values of <96.0% (value for *Rathayibacter rathayi* DSM 7485^T) with respect to other members of the suborder *Microbacteriaceae* used in the phylogenetic analysis.

Cell-wall analysis showed that the peptidoglycans of strains KSL-113^T and KSL-133 contained 2,4-diaminobutyric acid (DAB), alanine, glycine and glutamic acid in approximate ratios 1.5:0.8:1.8:1.0 and 1.9:0.9:1.8:1.0, respectively. The peptide Gly-D-Glu was present in partial peptidoglycan hydrolysates (4 M HCl; 45 min at 100 °C) of both strains, indicating the presence of B-type cross-linkage. From these data, it was concluded that strains KSL-113^T and KSL-133 have the peptidoglycan type B2_γ, based on D-Glu-D-DAB, with Gly in position 1 and L-DAB in position 3, as described by Schleifer & Kandler (1972). Strains KSL-113^T and KSL-133 contained MK-12, at peak area ratios of approximately 61 and 70%, respectively, as the predominant menaquinone; significant amounts (17–18%) of MK-11 were also present. The major polar lipids detected in strains KSL-113^T and KSL-133 were diphosphatidylglycerol, phosphatidylglycerol, unidentified phospholipids and unidentified glycolipids. Strains KSL-113^T and KSL-133 had cellular fatty acid profiles that contained large amounts of branched fatty acids: the major components (>10% of total fatty acids) were anteiso-C_{15:0}, iso-C_{16:0} and anteiso-C_{17:0} (see Supplementary Table S1 available in IJSEM Online). The DNA G+C contents of strains KSL-113^T and KSL-133 were 71.1 and 71.6 mol%, respectively.

Strains KSL-113^T and KSL-133 could be clearly distinguished from members of the family *Microbacteriaceae* by differences in chemotaxonomic properties. Strains KSL-113^T and KSL-133 differed from the genera *Microcella* and *Frigoribacterium*, the closest phylogenetic neighbours, in terms of the predominant menaquinone type and the diamino acid in position 3 of the peptidoglycan. Members of the genera *Microcella* and *Frigoribacterium* contain MK-12 plus MK-13 and MK-9 as the predominant menaquinones, respectively, and possess D-lysine as the diamino acid in position 3 of the peptidoglycan (Kämpfer *et al.*, 2000; Tiago *et al.*, 2005). Strains KSL-113^T and KSL-133 were also distinguishable from the genus *Microcella* by differences in the cellular fatty acid profiles, particularly with regard to the major fatty acids (Tiago *et al.*, 2005; Table 1 and Supplementary Table S1). Strains KSL-113^T and KSL-133 could be differentiated from other genera of the family *Microbacteriaceae* by differences in the predominant menaquinone and the diamino acid types (Kämpfer *et al.*, 2000; Manaia *et al.*, 2004; Lin *et al.*, 2004). Although the chemotaxonomic properties of strains KSL-113^T and KSL-133 were similar to those of members of the genus *Agromyces*, both strains exhibited a low level of 16S rRNA gene sequence similarity with respect to the type species of the genus *Agromyces* (Fig. 1). When the DNAs of strains KSL-113^T and KSL-133 were used individually as labelled DNA probes for cross-hybridization, the mean DNA–DNA relatedness value was found to be 92%, indicating that the

two strains were members of the same genomic species (Wayne *et al.*, 1987). In view of their combined phenotypic, phylogenetic and genetic similarities, strains KSL-113^T and KSL-133 can be considered as members of the same species. Therefore, on the basis of the data presented, strains KSL-113^T and KSL-133 should be classified within a novel genus and species, for which the name *Yonghaparkia alkaliphila* gen. nov., sp. nov. is proposed.

Description of *Yonghaparkia* gen. nov.

Yonghaparkia (Yong.ha.park'i.a. N.L. fem. n. *Yonghaparkia* named after Yong-Ha Park, a Korean microbiologist who proposed the family *Microbacteriaceae* and has contributed significantly to bacterial systematics).

Cells are Gram-positive, non-spore-forming short rods or rods (0.2–0.4 × 0.6–1.4 μm). Strictly aerobic. The cell-wall peptidoglycan type is B2_γ (based on D-Glu-D-DAB, with Gly in position 1 and L-DAB in position 3). The predominant menaquinone is MK-12; a significant amount of MK-11 is also present. The fatty acid profile consists of branched and straight-chain fatty acids. The DNA G+C content is 71.1–71.6 mol% (HPLC). The type species is *Yonghaparkia alkaliphila*.

Description of *Yonghaparkia alkaliphila* sp. nov.

Yonghaparkia alkaliphila (al.ka.li.phi'la. N.L. n. *alkali* alkali; Gr. adj. *philos* loving; N.L. fem. adj. *alkaliphila* loving alkaline conditions).

Colonies are circular, slightly convex, smooth, glistening, yellow in colour and 1.5–2.0 mm in diameter after incubation for 10 days at 30 °C on 2 × diluted NA (pH 9.0). Growth occurs at 10 and 37 °C, but not at 4 or 38 °C. Optimal pH for growth is 9.0. Growth occurs in the absence of NaCl and in the presence of 2% (w/v) NaCl, but not in the presence of 3% (w/v) NaCl. Growth does not occur under anaerobic conditions on 2 × diluted NA (pH 9.0) or on 2 × diluted NA (pH 9.0) supplemented with nitrate. Urease-negative. Starch and Tween 40 are hydrolysed, but hypoxanthine, xanthine, L-tyrosine and Tweens 20, 60 and 80 are not. Negative for nitrate reduction. In assays with the API ZYM system, esterase (C4), esterase lipase (C8), leucine arylamidase, β-glucuronidase and α-glucosidase are present, but alkaline phosphatase, lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase are absent. Acetate, succinate, benzoate, L-malate, formate and L-glutamate are not utilized as sole carbon and energy sources. Utilization of salicin is variable (positive for type strain). Susceptible to polymyxin B, streptomycin, chloramphenicol, gentamicin, novobiocin, lincomycin, oleandomycin, neomycin and carbenicillin, but not to penicillin G, ampicillin, tetracycline or kanamycin. The major fatty acids (>10% of total fatty acids) are

anteiso-C_{15:0}, iso-C_{16:0} and anteiso-C_{17:0}. The major polar lipids are diphosphatidylglycerol, phosphatidylglycerol, unidentified phospholipids and unidentified glycolipids. The DNA G + C content is 71.1–71.6 mol% (71.1 mol% for type strain) (HPLC).

The type strain, KSL-113^T (=KCTC 19126^T=CIP 108920^T), was isolated from an alkaline soil from Korea.

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