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NOTE

Leucobacter kyeonggiensis sp. nov., a New Species Isolated from Dye Waste Water[§]

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A Gram-positive, aerobic, non-motile bacterium designated F3-P9^T, was isolated from dye waste water in Korea and was characterized using a polyphasic taxonomic approach. Comparative 16S rRNA gene sequence analysis showed that strain F3-P9^T belongs to genus *Leucobacter*. The 16S rRNA gene sequence similarities among strain F3-P9^T and validated representatives of the genus *Leucobacter* ranged from 95.9-97.4%. Strain F3-P9^T exhibited DNA-DNA relatedness values below 48% with respect to *Leucobacter* species. The G+C content of the genomic DNA was 67.5 mol%. F3-P9^T contained MK-11 as the major respiratory quinone. The major fatty acids were anteiso-$C_{15:0}$ (48.5%), anteiso-$C_{17:0}$ (22.7%), and iso-$C_{16:0}$ (14.5%). The peptidoglycan was composed of L-2,4-diaminbutyric acid, alanine, glycine, and glutamic acid. The polar lipid profile showed a major amount of diphosphatidylglycerol (DPG), a moderate amount of phosphatidylglycerol (PG), and two unknown glycolipids. On the basis of its phenotypic and genotypic properties and its phylogenetic distinctiveness, strain F3-P9^T (KEMC 211-128^T =KACC 16572^T =JCM 17539^T) should be classified in the genus *Leucobacter* as the type strain of a novel species, for which the name *Leucobacter kyeonggiensis* sp. nov. is proposed.

***Keywords*:** Taxonomy, 16S rRNA gene, *Microbacteriaceae, Leucobacter kyeonggiensis*

The genus *Leucobacter* was described by Takeuchi *et al.* (1996). It is comprised of aerobic, non-motile rods with the cell wall containing 2,4-diaminobutyric acid (DAB) in a B-type cross-linked peptidoglycan; however, some members are also reported to contain γ-aminobutyric acid (GABA) in cell wall peptidoglycan. At the time of writing, the genus comprises 14 species and subspecies with validly published names: *Leucobacter komagatae* (Takeuchi *et al*., 1996), *L. albus* (Lin *et al.,* 2004), *L. aridicollis* (Morais *et al*., 2004), *L. chromiireducens* subsp. *chromiireducens* (Morais *et al*., 2004), *L. alluvii*, *L. luti* (Morais *et al*., 2006), *L. chromiireducens* subsp. *solipictus* (Muir and Tan, 2007), *L. iarius* (Somvanshi *et al*., 2007), *L. tardus* (Behrendt and Schumann, 2008), *L. chironomi* (Halpern *et al*., 2009), *L. aerolatus* (Martin *et al*., 2010), *L. chromiiresistens* (Sturm *et al*., 2011), *L. exalbidus* (Ue, 2011), and *L. celer* (Shin *et al.,* 2011).

In the course of screening microorganisms from dye waste water, a Gram-positive and non-motile strain F3-P9^T, was isolated. On the basis of 16S rRNA gene sequence analysis, strain F3-P9^T was considered to be a *Leucobacter*-like strain. Strain F3-P9^T was subjected to a polyphasic taxonomic investigation and the results indicated that strain F3-P9^T should be placed in the genus *Leucobacter* as a new species.

Strain F3-P9^T was originally isolated from an aeration tank of a textile dye wastewater treatment system in the Banwol industrial area, Gyeonggi-do, South Korea (37°18′00.48″N, 126°47′59.15″E, altitude 14 m). The physical conditions of dye wastewater were pH 7.0 and 35.5°C. One milliliter of water was serially diluted with saline (0.85% NaCl) solution, vortexed, and 100 μl of the aliquot was spread on ten times diluted R2A agar (Difco, USA). Single colonies on the plates were purified by transferring them onto new plates and incubating them once again under the same conditions. The isolates were routinely cultured on LB agar at 30°C and maintained as a glycerol suspension (20%, w/v) at -70°C. The closely related type strains of *Leucobacter* species were obtained from the Korean Agricultural Culture Collection (KACC, Korea) and Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Germany).

For the phylogenetic analysis of strain F3-P9^T, genomic DNA was extracted using a commercial genomic DNA extraction kit (Solgent, Korea) and PCR-mediated amplification of the 16S rRNA gene and the sequencing were carried out according to Kim *et al.* (2005). The nearly complete sequence of the 16S rRNA gene was compiled with SeqMan software (DNAStar, Inc., USA). The 16S rRNA gene sequences of the related taxa were obtained from GenBank and edited using the BioEdit program (Hall, 1999). Multiple alignments were performed with the CLUSTAL X program (Thompson *et al*., 1997). The evolutionary distances were calculated using the Kimura two-parameter model (Kimura, 1983). The phylogenic tree was constructed using the neighbor-joining method (Saitou and Nei, 1987) and maximum-parsimony (Fitch, 1971) in the MEGA3 Program (Kumar *et al.*, 2004). Furthermore, we conducted bootstrap analyses with 1,000 replicates to obtain confidence levels for the branches (Felsenstein, 1985) and con-

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structed a maximum-likelihood tree using the PHYLIP Program (Choi *et al*., 2000; Brinkman *et al*., 2001).

The Gram reaction was carried out according to the classical Gram procedure described by Doetsch (1981). The cell morphology and motility were examined by light microscopy (Nikon E600) and transmission electron microscopy (TEM). TEM was carried out as follows. Strain F3-P9^T was grown on LB agar at 30℃ for 18 h. Cells were gently resuspended in 1 drop of deionized water and samples were placed on carbon- and Formvar-coated nickel grids for 30 sec. Grids were floated on 1 drop of 0.1% (wt/vol) aqueous uranyl acetate, blotted dry, and then viewed with a Carl Zeiss LEO912AB electron microscope at 100 kV under standard operating conditions. Catalase and oxidase tests were performed as outlined by Cappuccino and Sherman (2002). Physiological characteristics were determined with API 20 NE, API ID 32 GN, API 50 CH and API ZYM galleries according to the instructions of the manufacturer (bioMérieux, France). Growth at different temperatures (4, 10, 15, 20, 30, 37, 42 and 50℃) and various pH values (pH 5.0-10.0 at intervals of 1 pH units) was assessed on Luria-Bertani broth (LB, Difco) after 5 days incubation. Salt tolerance was tested in Luria-Bertani broth supplemented with 0-10 % (w/v) NaCl incubated for 5 days at 30℃. Degradation of DNA (DNase agar, Scharlau), casein, starch and tween-80 (Atlas, 1993) was also investigated; reactions were read after 5min. Growth on nutrient agar (NA, Difco), trypticase soy agar (TSA, Difco) and Luria-Bertani agar was also evaluated at 30℃.

Isoprenoid quinones were extracted with chloroform/methanol (2:1, v/v), purified by TLC (thin-layer chromatography) and

Table 1. Differential phenotypic characteristics of strain F3-P9^T and closely related *Leucobacter* type strains
Strains: 1, Strain F3-P9 ^{T} 2, *Leucobacter celer* KACC 14220^T 3, *L. chromiiresistens* DSMZ 12440^T 4, *L. alluvii* KACC 21117^T 5, *L. chironomi* KACC 21124^T 6, *L. iarius* KACC 21116^T 7. *L. komagatae* KACC 25346^T.
Data were obtained in this study under identical growth conditions. Results are scored as: +, positive; -, negative; w, weakly positive.

Characteristic	1	2	3	4	5	6	7
Colony color	Cream	Cream	Yellow	Cream	Cream	Cream	Cream
Growth at							
4℃	+	+	+	+	-	-	-
42℃	+	+	+	+	-	-	+
45℃	-	+	-	-	-	-	-
8% NaCl	+	+	+	+	-	+	+
Optimum Growth							
Temperature (℃)	30℃	37℃	30℃	30℃	30℃	30℃	30℃
pH	8	8	7-8	7-8	7	7	7
NaCl (w/v)%	0	0	2	0	0-1	0	1
Enzyme activity							
DNase	-	-	-	+	-	-	-
N-Acetyl-β-glucosaminidase	-	-	-	-	-	+	-
Acid phosphatase	w	-	-	-	w	+	-
Arginine dihydrolase	+	-	-	-	-	-	-
Esterase (C4)	+	-	w	+	-	-	-
Esterase (C8)	w	-	-	-	+	-	+
β-Galactosidase (ONPG)	-	-	-	-	-	-	+
β-Glucosidase (Esculin hydrolysis)	-	+	-	-	-	-	+
β-Glucosidase	-	-	-	-	-	+	-
Leucine arylamidase	+	-	+	+	w	w	+
Naphtol-AS-BI-phosphohydrolase	w	-	+	+	+	w	+
Glycerol	-	+	+	+	-	+	+
Trypsin	+	-	+	-	+	-	w
Hyrolysis of							
Tween 80	-	+	+	+	-	-	+
Starch	-	-	+	-	-	-	-
Assimilation							
2-Ketogluconate (α)	-	-	-	+	-	-	-
Gluconate	+	-	-	-	-	-	-
L-Fucose	+	-	-	-	-	-	-
D-Mannose	-	+	-	-	-	-	-
D-Ribose	-	+	-	-	-	+	-
myo-Inositol	+	+	-	+	-	-	-
L-Alanine	-	-	+	+	+	+	-
L-Histidine	+	-	+	+	-	+	+
L-Proline	+	-	+	+	+	+	+

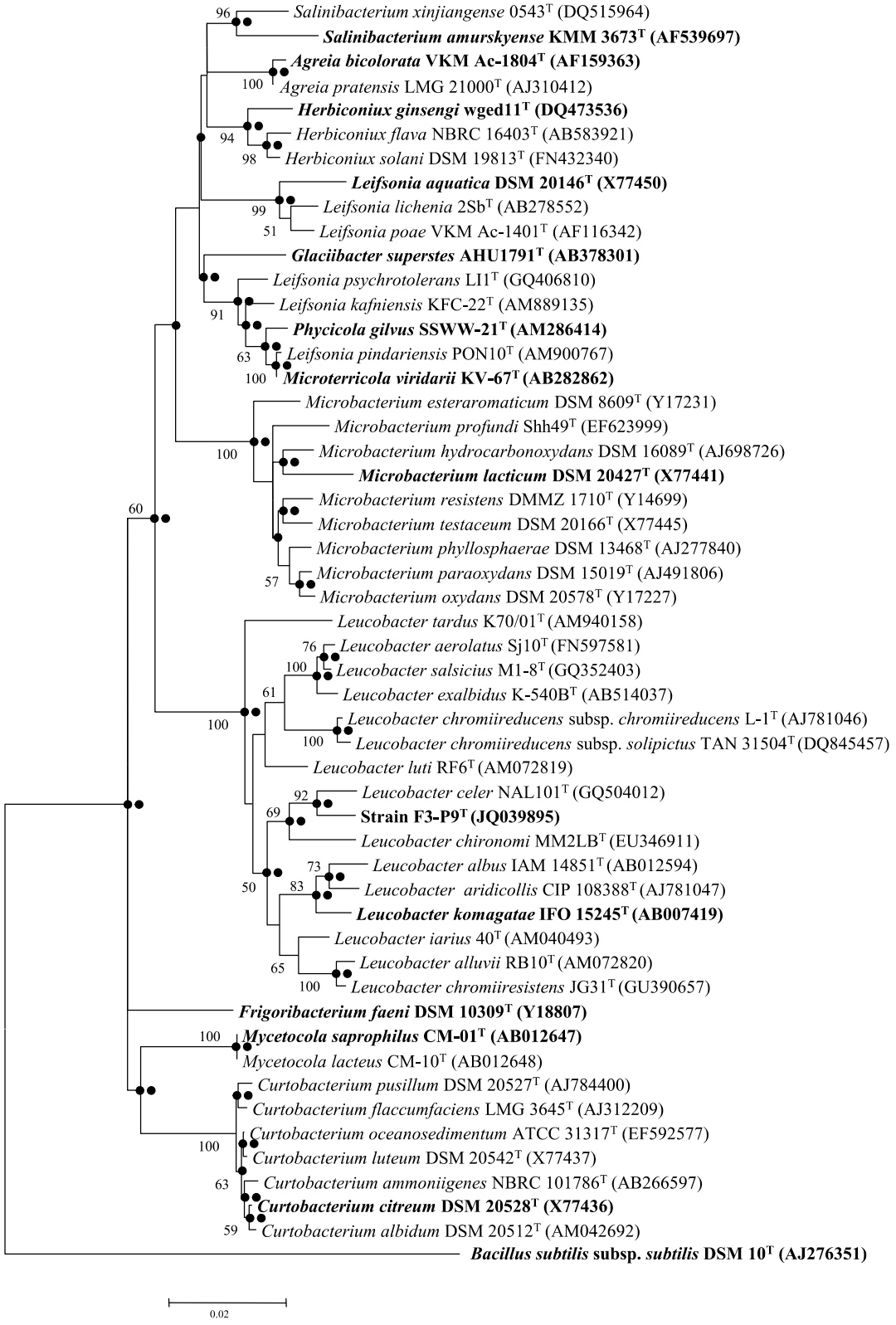

Fig. 1. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences, showing the relationships among strain F3-P9^{T}and closely related species of the family *Microbacteriaceae*. Bootstrap values >50% are shown at the branch points. A filled circle indicates nodes recovered in either maximum-parsimony or the maximum-likelihood algorithm. Filled double-circles indicate nodes recovered in both algorithms. Bold characters indicate the type strains of each genus. The bar indicates 0.02 substitutions per nucleotide position.

subsequently analyzed by HPLC, as described by Collins and Jones (1981) and Shin *et al*., (1996). In order to perform fatty acid methyl ester analysis, cells were allowed to grow on TSA for 48 h at 30°C, and then two loops of the well-grown cells were harvested. Fatty acid methyl esters were prepared, separated, and identified with the Sherlock Microbial Identification System (Sherlock version 6.1; TSBA6), produced by MIDI, Inc., Newark, DE., USA (Sasser, 1990). Polar lipids of strain F3-P9^T were extracted according to the procedures described by Minnikin *et al.* (1984). Extracted lipids were separated by two-dimensional TLC and identified by spraying with appropriate detection reagent (Minnikin *et al*., 1984; Komagata and Suzuki, 1987). Ninhydrin reagent (ninhydrin reagent 0.2% solution, Sigma, USA) was used to detect free amino groups containing lipids, zinzadze reagent (molybdenum blue spray reagent, 1.3%, Sigma) for phosphorus containing lipids and molybdophoshpolic acid (phosphomolybdic acid reagent, 10 wt% solution in ethanol, Sigma-Aldrich, USA) for total lipids. The cell wall composition was analyzed using one-dimensional thin layer chromatography on cellulose sheets (TLC, Merck, Germany) as described by Schleifer and Kandler (1972) instead of paper chromatography. L-2, 4-diaminobutyric acid (L-DAB), γ-aminobutyric acid (GABA), alanine, glutamic acid, glycine, threonine, and aspartic acid were used as standard amino acids to analyze the amino acid composition of strain F3-P9^T and compared with reference strains.

For the determination of the DNA G+C content, the genomic DNA was extracted and purified with the QIAGEN Genomic-tip system 100/G (QIAGEN, Germany) and was then enzymatically degraded into nucleosides. The nucleosides were analyzed using HPLC as described previously (Tamaoka and Komagata, 1984; Mesbah *et al*., 1989).

The DNA-DNA hybridization was performed fluorometrically, according to the method developed by Ezaki *et al*. (1989), using photobiotin-labelled DNA probes and micro-dilution wells. The hybridization was conducted in five replications for each sample. The highest and lowest values obtained for each sample were excluded. The remaining three values were utilized in the calculation of hybridization values.

Strain F3-P9^T was Gram-positive, aerobic, non-motile, and rod-shaped (0.7-0.9 μm wide and 1.7-2.0 μm long). (See Supplementary Fig. S1). Phenotypic and biochemical characteristics that differentiate strain F3-P9^T from other *Leucobacter* species are listed in Table 1.

A nearly complete 16S rRNA gene sequence of strain F3-P9^T (1432 nt) was obtained. On the basis of 16S rRNA gene sequence similarity, the most closely related strains were *Leucobacter celer* NAL101^T (98.4%), *Leucobacter chromiiresistens* JG31^T (97.5%), *Leucobacter alluvii* RB10^T (97.4%), *Leucobacter chironomi* MM2LB^T (97.2%) and *Leucobacter iarius* 40^T (97.2%). In the neighbor-joining phylogenetic tree (Fig. 1), strain F3-P9^T was clustered with *Leucobacter celer* NAL101^T with a bootstrap value of 92% and this clade joined the cluster comprising members of the genus *Leucobacter,* this was also confirmed in the maximum-parsimony and maximum-likelihood algorithm (See Supplementary Fig S2 and S3).

Strain F3-P9^T contained menaquinone MK-11 as the major respiratory quinone (53.6%) and moderate amounts of MK-9 (24%) and MK-10 (28%) were also detected. Strain F3-P9^T contained a higher content of anteiso-$C_{15:0}$ (48.5%), anteiso-$C_{17:0}$ (22.7%), iso-$C_{16:0}$ (14.5%), and moderate amounts of iso-$C_{15:0}$ (9.9%) and iso-$C_{17:0}$ (2.9%). These fatty acid profiles have the general characteristics of genus *Leucobacter*, although there were differences in the proportions of fatty acids between species, which are listed in Table 2. The hydrolysate of the peptidoglycan of the strain F3-P9^T was composed of L-DAB, alanine, glycine, and glutamic acid. The identified strain F3-P9^T possesses a major polar lipid component of diphosphatidylglycerol (DPG), moderate amounts of phosphatidylglycerol (PG) and two unknown glycolipids. A trace amount of an unknown aminolipid was also detected. (See Supplementary Fig S4). The polar lipid profile aligns with the reported profiles of other *Leucobacter* species. Most species of the genus *Leucobacter* are high G+C mol containing bacteria, ranging from 62.8 to 69.4%. The DNA G+C content of strain F3-P9^T was 67.5 mol%.

To differentiate strain F3-P9^T from closely related species, DNA-DNA hybridization was performed. The DNA relatedness values are expressed as means of three values. Strain F3-P9^T exhibited low levels of DNA relatedness with *L. celer* KACC 14220^T (28±3), *L. chromiiresistens* DSMZ 12440^T (19±1), *L. alluvii* KACC 21117^T (10±4), *L. chironomi*KACC 21124^T (4±0), *L. iarius* KACC 21116^T (8±1), and *L. komagatae* KACC 25346^T (7±0). (See Supplementary table S1). The DNA-DNA hybridization values with other members of the *Leucobacter* were less than 15%. These values indicate that strain F3-P9^T is not related to them at the species level (Wayne *et al*., 1987).

Phylogenetic and chemotaxonomic characteristics of strain F3-P9^T were compared with those of closely related *Leucobacter* and found to be significantly differentiated from those of phy-

Table 2. Cellular fatty acid profiles of strain F3-P9 ^{T} and closely related species
Strains: 1, Strain F3-P9 ^{T} 2, *Leucobacter celer* KACC 14220^T 3, *L. chromiiresistens* DSMZ 12440^T 4, *L. alluvii* KACC 21117^T 5, *L. chironomi* KACC 21124^T 6, *L. iarius* KACC 21116^T 7. *L. komagatae* KACC 25346^T.
Validated type strains were grown on TSA at 30°C for 2 days for fatty acid analysis and all the data were obtained in the present study. Fatty acids that were less than 1.0% of the total fatty acids are noted as trace (Tr).

Fatty acids	1	2	3	4	5	6	7
14:0 iso	Tr	2.5	Tr	Tr	3.7	Tr	3.8
15:0 iso	9.9	9.8	9.7	1.0	8.4	3.7	9.0
15:0-anteiso	48.5	46.5	52.8	59.0	58.9	54.7	57.1
16:0 iso	14.5	16.1	14.2	11.7	16.5	14.5	16.8
16:00	Tr	1.6	3.7	2.1	1.3	Tr	1.6
17:0 iso	2.9	Tr	Tr	Tr	Tr	Tr	Tr
17:0-anteiso	22.7	21.1	18.3	25.5	10.4	24.6	10.7

logenetically related *Leucobacter* species. Strain F3-P9^T was differentiated from the closely related members by its ability to produce arginine dihydrolase and by assimilation of gluconate and L-fucose. Moreover, the low DNA-DNA relatedness also supports the result of the polyphasic analysis. Based on these results, strain F3-P9^T represents a new species within the genus *Leucobacter* for which the name *Leucobacter kyeonggiensis* sp. nov. is proposed.

Description of *Leucobacter kyeonggiensis* sp. nov.

Leucobacter kyeonggiensis (kye.ong.gi.en'sis. N.L. masc. adj. kyeonggiensis, pertaining to Kyeonggi Province, South Korea, from where the type strain was isolated).

Cells were Gram-positive, aerobic, non-motile, 0.7-0.9 μm in width and 1.7-2.0 μm in length. After 3 days on R2A, colonies were 1-2 mm in diameter, smooth, creamy colored, circular and convex. Catalase activity was detected but oxidase was absent. It growed between 4 and 42°C and the optimum growth temperature is 30°C on LB broth. The pH growth range was between pH 6.0 and 10.0, with an optimum pH 6.0 in LB broth. It tolerates up to 10% NaCl with optimum growth observed at 0% NaCl. It does not reduced nitrate to nitrogen gas. Growth occurred on R2A agar, nutrient agar, TSA, and LB agar. Starch, DNA, casein and Tween 80 were not hydrolyzed.

Cells showed activities for acid phosphatase, arginine dihydrolase, α-chymotrypsin, esterase (C4), cystein arylamidase, esterase (C8), leucine arylamidase, naphtol-AS-BI-phosphohydrolase, protease (gelatin hydrolysis) and trypsin. Cells showed no activities for *α*-fucosidase, *α*-galactosidase, *α*-glucosidase (starch hydrolysis), *α*-mannosidase, *β*-galactosidase (ONPG), *β*-glucosidase, *β*-glucosidase (esculin hydrolysis), lipase (C14), *N*-acetyl-*β*-glucosaminidase, and urease.

Acid was produced from D-fructose, esculin ferric citrate, gluconate, inositol and L-rhamnose. Acid was not produced from 2-ketogluconate, 5-ketogluconate, *α*-methyl-D-glucoside, *α*-methyl-D-mannoside, amygdalin, arbutin, *β*-methyl-D-xyloside, dulcitol (galactitol), D-adonitol (Ribitol), D-arabinose, D-arabitol, D-cellobiose, D-fucose, D-galactose, D-mannose, D-raffinose, D-sucrose, D-tagatose, D-trehalose, D-xylose, erythritol, glycerol, glycogen, L-arabitol, L-arabinose, L-xylose, mannitol, melezitose, ribose, sorbitol, turanose and xylitol.

The following substrates were utilized for growth: 3-hydroxybenzoate, D-maltose, gluconate, L-histidine, L-proline, L-rhamnose, *myo*-inositol, and protease. The following substrates were not utilized for growth: 2-ketogluconate, 5-ketogluconate, acetate, capric acid, citrate, D-glucose, D-maltose, D-mannitol, D-melibiose, D-ribose, D-sorbitol, glycogen, itaconic acid, lactate, L-alanine, L-arabinose, L-fructose, L-serine, malonate, N-actetyl-glucosamine, propionic acid, salicin, suberic acid, and valeric acid.

The major fatty acids were anteiso-$C_{15:0}$ (48.5%), anteiso-$C_{17:0}$ (22.7%), and iso-$C_{16:0}$ (14.5%). The peptidoglycan was composed of L-DAB, alanine, glycine and glutamic acid. The polar lipid profile showed major amounts of phosphatidylglycerol (PG) and diphosphatidylglycerol (DPG). MK-11 was the major respiratory menaquinone (53.6%), with moderate amounts of MK-9 (24%) and MK-10 (28%) also detectable. DNA G+C content was 67.5 mol%. The type strain, F3-P9^T (KEMC 211-128^T =KACC 16572^T =JCM 17539^T) was isolated from dye waste water of Ansan City, Republic of Korea.

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The NCBI GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain F3-P9^T is JQ039895.

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