Runella defluvii sp. nov., isolated from a domestic wastewater treatment plant

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A long, Gram-negative, rod-shaped bacterium, designated strain EMB13^T, was isolated from a wastewater treatment plant in Korea. The isolate was strictly aerobic and non-motile. The strain grew optimally at 30–35 °C and pH 7.5–8.0, and the predominant fatty acids were iso-C_{15:0}, summed feature 3 (C_{16:1ω7c} and/or iso-C_{15:0} 2-OH), C_{16:1ω5c} and iso-C_{17:0} 3-OH. The strain contained a large amount of phosphatidylethanolamine and small amounts of phosphatidylcholine and an unknown phospholipid as the polar lipids. The G+C content of the genomic DNA was 40.1 mol% and the major isoprenoid quinone was menaquinone-7. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain EMB13^T belonged to the genus *Runella* and was most closely related to *Runella limosa* EMB111^T, with a 16S rRNA gene sequence similarity of 97.1 %. DNA–DNA relatedness between strain EMB13^T and *R. limosa* EMB111^T was approximately 25 %. On the basis of phenotypic, chemotaxonomic and molecular data, it is clear that strain EMB13^T represents a novel species within the genus *Runella*, for which the name *Runella defluvii* sp. nov. is proposed. The type strain is EMB13^T (=KCTC 12614^T =DSM 17976^T).

The genus *Runella*, a member of the family ‘*Flexibacteraceae*’, was first proposed by Larkin & Williams (1978). Currently, the genus contains three species, *Runella slithyformis* (Larkin & Williams, 1978), *Runella zae* (Chelius *et al.*, 2002) and *Runella limosa* (Ryu *et al.*, 2006). *R. zae* was isolated from the stems of surface-sterilized *Zea mays*; in contrast, *R. slithyformis* and *R. limosa* were isolated from aquatic habitats, suggesting that aquatic environments may be important habitats for the genus *Runella*. Activated sludge processes have been used to remove organic compounds as well as nutrients from wastewater, and insight into the bacterial communities is a prerequisite for understanding activated sludge processes. Therefore, efforts have been made in our laboratory to isolate and characterize members of the bacterial community in activated sludge (Lu *et al.*, 2006; Park *et al.*, 2006, 2007). In this study, we describe another novel species belonging to the genus *Runella* isolated from an activated sludge process treating domestic sewage.

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain EMB13^T is DQ372980.

A transmission electron micrograph of cells of strain EMB13^T and API 50CH utilization results for strain EMB13^T are available as supplementary material with the online version of this paper.

Strain EMB13^T was isolated from activated sludge of a domestic wastewater treatment plant in Pohang, Korea. A sludge sample was serially diluted with 1 % (w/v) saline solution, spread on R2A agar (Difco) and incubated at 20 °C for 5 days. Subcultivation was done on R2A agar at 30 °C for 3 days.

Gram staining was performed using the bioMérieux Gram stain kit according to the instructions of the manufacturer. Cell morphology and motility were studied using phase-contrast microscopy and transmission electron microscopy (JEM-1010; JEOL) as described by Jeon *et al.* (2005). Physiological characteristics of strain EMB13^T were examined by growing the isolate on R2A agar at different temperatures (5–50 °C at 5 °C intervals) and in R2A broth adjusted at different pH values (pH 5.0–10.0 at 0.5 pH unit intervals) (Gomori, 1955). Oxidase activity was tested by oxidation of 1 % (w/v) tetramethyl-*p*-phenylenediamine (Merck) and catalase activity was evaluated by production of oxygen bubbles in 3 % (v/v) aqueous hydrogen peroxide solution. Hydrolysis of tyrosine, Tweens 80 and 20, aesculin, casein, starch, gelatin and urea was assessed on R2A agar after 5 days incubation according to methods described previously (Lanyi, 1987; Smibert & Krieg, 1994). Nitrate reduction was assessed according to the method of Lanyi (1987) and acid production from carbohydrates was

tested as described by Leifson (1963). Additional enzyme activities and biochemical features were determined using API ZYM, API 20E and API 50CH kits as recommended by the manufacturer (bioMérieux) except that kits were incubated at 30 °C. Strain EMB13^T on R2A agar formed slightly raised, circular, salmon-coloured colonies when grown at 30 °C for 3 days. Growth was observed at temperatures between 15 and 40 °C, with an optimum growth temperature of 30–35 °C. The strain grew at pH 6.0–9.5, with an optimum of pH 7.5–8.0. Cells of the isolate were non-motile rods (0.5–0.9 µm wide and 2.2–6.0 µm long) without flagella (Supplementary Fig. S1 available in IJSEM Online). Cells of strain EMB13^T were Gram-negative, oxidase-negative and catalase-positive and did not reduce nitrate to nitrite. The isolate was negative for production of indole, H₂S and acetoin and citrate utilization (API 20E). Anaerobic growth was not observed under anaerobic conditions over a 7 day incubation at 30 °C on R2A agar.

Analysis of fatty acid methyl esters was performed according to the instructions of the Microbial Identification System (MIDI; Microbial ID, Inc.). The DNA

G + C content of strain EMB13^T was determined using an HPLC fitted with a reversed-phase column (GROM-SIL 100 ODS-2FE; GROM) according to the method of Tamaoka & Komagata (1984). Analyses of polar lipids and isoprenoid quinones were carried out using the methods described by Komagata & Suzuki (1987). The major respiratory lipoquinone of strain EMB13^T was menaquinone-7 (MK-7). The isolate contained iso-C_{15:0} (29.0%), summed feature 3 (C_{16:1}ω7c and/or iso-C_{15:0} 2-OH; 20.2%), C_{16:1}ω5c (10.8%) and iso-C_{17:0} 3-OH (9.2%) as the major fatty acids, which is similar to the profiles of other *Runella* species (Table 1). The G + C content of the genomic DNA was 40.1 mol%. The polar lipid profile of strain EMB13^T was dominated by a large amount of phosphatidylethanolamine, and small amounts of phosphatidylcholine and an unknown phospholipid (PL1) were also present, which shows that the isolate can be clearly differentiated from *R. limosa* EMB111^T (Table 1). Typical phenotypic characteristics of strain EMB13^T are summarized and compared with those of the type strains of closely related taxa in Table 1 and Supplementary Table S1. Some properties, such as the fatty acid composition, major

Table 1. Differential phenotypic characteristics of strain EMB13^T and selected related species

Strains/species: 1, strain EMB13^T (data from this study); 2, *R. limosa* EMB111^T (Ryu *et al.*, 2006); 3, *R. slithyformis* (Chelius & Triplett, 2000); 4, *R. zeeae* NS12^T (Chelius *et al.*, 2002); 5, *Dyadobacter fermentans* NS114^T (Chelius & Triplett, 2000; Reddy & Garcia-Pichel, 2005). +, Positive; –, negative; w, weak reaction; NA, not available. All taxa are negative for nitrate reduction and gelatin hydrolysis.

Characteristic	1	2	3	4	5
Morphology	Rods	Filamentous	Curved rods	Bent rods	Rods
Colony colour	Salmon	Salmon	Salmon	Salmon	Yellow
Optimum temperature (°C)	30–35	25–30	20–30	28	28
Oxidase	–	–	+	+	+
Catalase	+	+	w	+	+
Starch hydrolysis	–	–	w	–	–
Acid produced from glucose	–	+	–	+	+
Sole carbon sources for growth*					
Glycerol	+	–	–	–	w
D-Arabitol	–	–	w	–	+
L-Arabitol	–	–	–	–	w
Dulcitol	–	–	w	w	w
Inositol	–	–	w	w	+
Mannitol	–	–	w	–	+
Sorbitol	–	–	w	w	w
Glycogen	w	w	+	+	–
Sorbose	w	w	w	w	+
5-Ketogluconate	w	+	–	w	+
Methyl β-xyloside	+	–	+	+	+
Maltose	–	+	+	+	+
Trehalose	–	+	+	+	+
Major cellular fatty acids	iso-C _{15:0} , C _{16:1} ω5c, SF3†	iso-C _{15:0} , C _{16:1} ω5c, SF3	NA	C _{16:1} ω5c, iso-C _{15:0} , SF3	iso-C _{15:0} , C _{16:1} ω5c, C _{16:1} ω7c
Major polar lipid‡	PE	PG	NA	NA	NA
DNA G + C content (mol%)	40.1	44.5	49	49	48

*Results for *R. limosa* EMB111^T were obtained in this study.

†SF3, Summed feature 3, containing C_{16:1}ω7c and/or iso-C_{15:0} 2-OH.

‡PE, Phosphatidylethanolamine; PG, phosphatidylglycerol.

lipoquinone and nitrate reduction, are in accordance with those of members of the genus *Runella*, whereas some others such as DNA G+C content and major polar lipids allow the differentiation of strain EMB13^T from closely related species.

Sequencing of the 16S rRNA gene of strain EMB13^T was carried out as described previously (Lane, 1991). The PCR product was cloned using a TOPO cloning kit (Invitrogen) and sequenced. The resulting 16S rRNA gene sequence (1427 nucleotides) was compared with available 16S rRNA gene sequences from GenBank using the BLAST program (<http://www.ncbi.nlm.nih.gov/BLAST/>) to determine an approximate phylogenetic affiliation, and gene sequences were aligned with those of closely related species by using the CLUSTAL W software (Thompson *et al.*, 1994). Phylogenetic trees were constructed by using three different algorithms, neighbour-joining, maximum-likelihood and maximum-parsimony algorithms; these methods are available in PHYLIP software, version 3.6 (Felsenstein, 2002). Sequence similarity values between the novel strain and related organisms were computed using the FASTA3 program in EBI (<http://www.ebi.ac.uk/fasta33/nucleotide.html>). A bootstrap analysis was performed according to the Kimura two-parameter model (Kimura, 1980) of the neighbour-joining method in the PHYLIP package. DNA–DNA hybridization was carried out to evaluate the genomic DNA relatedness between strain EMB13^T and *R. limosa* EMB111^T using the fluorometric microplate method (Ezaki *et al.*, 1989). Fluorometric data recorded after 30 min incubation were used for the calculation of a DNA–DNA hybridization value; the DNA relatedness value is the mean of three values.

Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain EMB13^T formed a phylogenetic lineage with *R. limosa* EMB111^T with 100% bootstrap value (Fig. 1). The overall topologies of the maximum-likelihood and maximum-parsimony trees were essentially the same as that of the neighbour-joining tree (data not shown). Comparative 16S rRNA gene sequence analysis showed that strain EMB13^T was most closely related to *R. limosa* EMB111^T, *R. zeae* NS12^T and *R. slithyformis* ATCC 29530^T, with sequence similarities of 97.1, 93.9 and 93.6%, respectively. The DNA–DNA relatedness between strain EMB13^T and *R. limosa* EMB111^T was about 25%, which is clearly below the 70% threshold generally accepted for species delineation (Stackebrandt *et al.*, 2002). The physiological, biochemical and phylogenetic properties of strain EMB13^T suggest that the isolate should be considered as representing an additional novel species belonging to the genus *Runella*, for which the name *Runella defluvii* sp. nov. is proposed.

Description of *Runella defluvii* sp. nov.

Runella defluvii (de.flu'vi.i. L. gen. n. *defluvii* of sewage).

Colonies are slightly raised, circular and salmon pink in colour on R2A agar. Cells are Gram-negative, non-motile

Fig. 1. Neighbour-joining tree showing the phylogenetic relationships based on 16S rRNA gene sequences of strain EMB13^T and related taxa. Bootstrap values are shown as percentages of 1000 replicates when greater than 50%. *Escherichia coli* ATCC 11775^T was used as an outgroup. Bar, 0.1 changes per nucleotide position.

rods, 0.5–0.9 µm wide and 2.2–6.0 µm long at 30 °C on R2A agar. Growth occurs at 15–40 °C (optimum, 30–35 °C) and at pH 6.0–9.5 (optimum, pH 7.5–8.0). Nitrate is not reduced to nitrite. Catalase-positive and oxidase-negative. No anaerobic growth after 7 days at 30 °C on R2A agar. Tyrosine, Tween 80 and aesculin are hydrolysed, but casein, Tween 20, starch, gelatin and urea are not hydrolysed. Acid is produced from raffinose, *myo*-inositol, lactose, L-arabinose, D-galactose, D-mannose, D-mannitol and melibiose, but not from D-glucose, D-fructose, arbutin or salicin. Indole, H₂S and acetoin are not produced and citrate is not utilized (API 20E). Alkaline phosphatase, trypsin, α-chymotrypsin, N-acetyl-β-glucosaminidase and naphthol-AS-BI-phosphohydrolase are produced, but tryptophan deaminase, esterase (C4), lipase (C14) and β-glucuronidase are not produced. Weak activities are observed for esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase, α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase, α-mannosidase and α-fucosidase (API ZYM). Utilizes glycerol, methyl β-xyloside, methyl α-D-mannoside and aesculin as sole carbon sources but not erythritol, D- or L-arabinose, D-xylose, adonitol, galactose, D-glucose, D-fructose, mannose, dulcitol, inositol, mannitol, sorbitol, N-acetylglucosamine, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, inulin, melezitose, D-raffinose, D-turanose, D- or L-arabitol, gluconate or 2-ketogluconate. Weakly utilizes ribose, L-xylose, sorbose, rhamnose, methyl α-D-glucoside, amygdalin, arbutin, starch, glycogen, xylitol, β-gentiobiose, D-lyxose, D-tagatose, D- and L-fucose and 5-ketogluconate (API 50CH). Contains a large amount of phosphatidylethanolamine and small amounts of phosphatidylcholine and an unknown phospholipid as polar lipids. The major isoprenoid quinone is menaquinone-7. The cellular fatty acids are

iso-C_{15:0} (29.0 %), summed feature 3 (C_{16:1}ω7c and/or iso-C_{15:0} 2-OH; 20.2 %), C_{16:1}ω5c (10.8 %), iso-C_{17:0} 3-OH (9.2 %), iso-C_{15:0} 3-OH (7.4 %), C_{15:0} (6.5 %), iso-C_{15:1} G (3.4 %), C_{16:0} 3-OH (3.3 %), iso-C_{13:0} (2.2 %), C_{15:1}ω6c (1.8 %), C_{16:0} (1.4 %), C_{17:1}ω6c (0.9 %), C_{14:0} (0.8 %), anteiso-C_{15:0} (0.6 %) and unknown ECL 14.959 (2.7 %). The G+C content of the DNA is 40.1 mol% (HPLC).

The type strain is EMB13^T (=KCTC 12614^T =DSM 17976^T), isolated from activated sludge of a domestic wastewater treatment plant.

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