Parapusillimonas granuli gen. nov., sp. nov., isolated from granules from a wastewater-treatment bioreactor

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A novel betaproteobacterium, designated strain Ch07^T, was isolated from granules from the wastewater-treatment bioreactor of an alcohol fermentation factory in South Korea. In order to determine its taxonomic position, the novel strain was characterized using a polyphasic approach. The new strain was Gram-negative, facultatively anaerobic, non-spore-forming, motile and short rod-shaped. 16S rRNA gene sequence analysis revealed that strain Ch07^T belonged to the class *Betaproteobacteria*, being related to *Pusillimonas noertemannii* BN9^T (gene sequence similarity 97.30 %), *Achromobacter xylosoxidans* subsp. *xylosoxidans* DSM 10346^T (97.09 %), *Bordetella pertussis* DSM 5571^T (97.01 %), *Pigmentiphaga kullae* DSM 13608^T (96.68 %) and *Castellaniella defragrans* DSM 1214^T (96.47 %). The results of DNA–DNA hybridization tests showed that reassociation values were less than 62 % with respect to these closely related type strains. Chemotaxonomic data showed that strain Ch07^T possessed ubiquinone Q-8. The G + C content of the genomic DNA was 67.9 ± 0.1 mol%. The major polyamine of strain Ch07^T was putrescine. The major polar lipids of strain Ch07^T were phosphatidylethanolamine, followed by diphosphatidylglycerol and phosphatidylglycerol. When strain Ch07^T was incubated on tryptic soy agar, the major cellular fatty acids were C_{16:0}, C_{17:0} cyclo, summed feature 3 (C_{16:1ω7c}/iso C_{15:0} 2-OH) and summed feature 5 (C_{18:1ω7c/ω9t/ω12t}). The results of DNA–DNA hybridizations, in combination with the chemotaxonomic and physiological data, demonstrated that strain Ch07^T represents a novel species of a new genus, for which the name *Parapusillimonas granuli* gen. nov., sp. nov. is proposed. The type strain of the type species is Ch07^T (=KCTC 12668^T=LMG 24012^T).

The family *Alcaligenaceae* is classified in the order of *Burkholderiales* in the class *Betaproteobacteria* and includes 17 genera, including *Pusillimonas*, *Bordetella*, *Achromobacter* and *Alcaligenes* (De Ley *et al.*, 1986; Busse & Auling, 1992).

Brownish-black granules (about 2 mm in diameter) from an upflow anaerobic sludge blanket (UASB) reactor, located in

Abbreviations: DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol.

The GenBank/EMBL/DBJ accession number for 16S rRNA gene sequence of strain Ch07^T is DQ466075.

Supplementary figures showing the production of fimbriae by strain Ch07^T, transmission electron micrographs of cells of strain Ch07^T and related strains, and the results of TLC analyses of polar lipids are available with the online version of this paper.

Daejeon, Korea, were homogenized by using an Ace Homogenizer (Nihonseiki Co.). The suspension was spread on R2A agar plates (Difco) after being serially diluted with 50 mM phosphate buffer (pH 7.0). The purified colonies were tentatively identified by partial sequences (1449 bp) of the 16S rRNA gene. Strain Ch07^T was one of the isolates that appeared on the plates under aerobic conditions. In this study, strain Ch07^T was investigated using a polyphasic taxonomic approach, including a phylogenetic analysis based on 16S rRNA gene sequences, DNA–DNA hybridization and chemotaxonomic and phenotypic properties. The results revealed that strain Ch07^T could be assigned to a new genus in the family *Alcaligenaceae*.

Cell morphology and motility were observed using a light microscope (× 1000), with the cells being allowed to grow

for 3 days at 30 °C on R2A agar. Colonies grown on R2A agar plates for 3 days were smooth and circular. For morphological observation with transmission electron microscopy, cells were grown on Luria–Bertani (LB; Difco LB broth) agar at 30 °C for 18 h and suspended bacterial cells were placed on carbon/Formvar-coated nickel grids for 30 s. Grids were floated on one drop of 0.1 % (w/v) aqueous uranyl acetate, blotted dry and then observed with a Carl Zeiss LEO912AB electron microscope at 100 kV under standard operating conditions. The Gram reaction was conducted according to the non-staining method as described by Buck (1982). Oxidase activity was evaluated via the oxidation of 1 % *p*-aminodimethylaniline oxalate and catalase activity was determined by measurements of bubble production after the application of 3 % (v/v) hydrogen peroxide solution (Cappuccino & Sherman, 2002). Growth at various temperatures (4, 15, 25, 30, 37 and 42 °C) was assessed on R2A agar. Growth at different pH values (pH 5.0–11.0, at intervals of 0.5 pH units) was assessed in R2A broth. Growth on nutrient agar (NA), LB agar and tryptic soy agar (TSA; BD) was also evaluated at 30 °C. The API 20NE, API ID32 GN, API 50CH and API ZYM microtest systems were conducted at 30 °C according to the manufacturer's recommendations (bioMérieux). Anaerobic growth was determined on R2A agar supplemented with thioglycolate (0.1 %, w/v) incubated at 28 °C for 10 days in a commercial system (GasPak EZ Gas Generating system, BD). Measurement of H₂S production was conducted on triple sugar–iron agar (BBL) at 28 °C for 6 days.

Isoprenoid quinones were extracted with chloroform/methanol (2:1, v/v), purified by TLC and subsequently analysed by HPLC, as previously described by Collins & Jones (1981) and Shin *et al.* (1996). For fatty acid analysis, strain Ch07^T and the type strains of related species (*Pusillimonas noertemannii* BN9^T, *Achromobacter xylosoxidans* subsp. *xylosoxidans* DSM 10346^T, *Bordetella pertussis* DSM 5571^T) were all incubated on TSA for 48 h at 28 °C. Even though *Pusillimonas noertemannii* BN9^T grew more slowly than strain Ch07^T and showed only weak growth on TSA, the same amount of biomass (50 mg) was collected for fatty acid analysis. Fatty acid methyl esters were identified with the Sherlock Microbial Identification System (MIDI) (Sasser, 1990). Polar lipids were extracted with chloroform/methanol (2:1, v/v) and a 10 µl sample was loaded on to the corners of two 10 × 10 cm thin layer silica gel 60 plates (Merck) according to Bligh & Dyer (1959). Polyamines were extracted and analysed according to Busse & Auling (1988) and Schenkel *et al.* (1995).

DNA–DNA hybridizations were performed with photobiotin labelled probes in microplate wells as described by Ezaki *et al.* (1989), using an HTS7000 Bio Assay Reader (Perkin Elmer) for the fluorescence measurements. The hybridization temperature was 51 °C. Reciprocal hybridization tests were performed in order to confirm the accuracy of the data. The highest and lowest values obtained for each sample were excluded and the means

of the remaining three values are quoted as the final DNA–DNA hybridization values.

For the determination of the DNA G + C content, genomic DNA was extracted and purified with the Qiagen Genomic-tip system 100/G (Qiagen) and was then degraded into nucleosides by P1 nuclease. The nucleosides were analysed using HPLC as described previously by Tamaoka & Komagata (1984) and Mesbah *et al.* (1989).

The 16S rRNA gene was amplified from the chromosomal DNA using the universal bacterial primer set, fD1 and rP1 (Weisburg *et al.*, 1991) and the purified PCR product was sequenced by Genotec, Daejeon, Korea (Kim *et al.*, 2005). The partial sequence of the 16S rRNA gene was compiled with SeqMan software (DNASTAR Inc.). 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). Evolutionary distances were calculated using the Kimura two-parameter model (Kimura, 1983). Phylogenetic trees were constructed by the neighbour-joining method (Saitou & Nei, 1987) and with the maximum-parsimony method (Fitch, 1971) in the MEGA3 program (Kumar *et al.*, 2001). A maximum-likelihood tree was also constructed using PHYLIP software (version 3.57c; Felsenstein, 1995). Bootstrap analysis with 1000 replicates was also performed in order to obtain confidence levels for the branches.

The 16S rRNA gene sequence of strain Ch07^T, a continuous stretch of 1449 nucleotides, was sequenced. Strain Ch07^T was found to belong to the family *Alcaligenaceae*. A search using the EzTaxon server (Chun *et al.*, 2007) showed that it was closely related to *Pusillimonas noertemannii* BN9^T (gene sequence similarity 97.30 %), *Achromobacter xylosoxidans* subsp. *xylosoxidans* DSM 10346^T (97.09 %), *Bordetella pertussis* DSM 5571^T (97.01 %), *Pigmentiphaga kullae* DSM 13608^T (96.68 %) and *Castellaniella defragrans* DSM 12141^T (96.47 %). A closer relationship between strain Ch07^T and members of the genera *Bordetella*, *Achromobacter*, *Pigmentiphaga*, *Castellaniella* and *Pusillimonas* (Fig. 1) was suggested by the sequence comparison programs.

The DNA–DNA relatedness values for strain Ch07^T were relatively low with the type strains of *Pusillimonas noertemannii* (27.4 %), *Achromobacter xylosoxidans* subsp. *xylosoxidans* (44.7 %) and *Bordetella pertussis* (61.9 %). Reciprocal hybridization tests resulted in DNA–DNA relatedness values of 35.3, 38.5 and 57.8 % with *Pusillimonas noertemannii*, *Achromobacter xylosoxidans* subsp. *xylosoxidans* and *B. pertussis*, respectively. These results confirmed that strain Ch07^T was a separate species at the genotype level according to the limits suggested by Wayne *et al.* (1987).

Members of the genera *Alcaligenes*, *Achromobacter* and *Pigmentiphaga* have DNA G + C contents ranging from 54 to 69 mol%. In contrast, members of the genera *Oligella* and *Taylorella* have relatively low G + C contents of only

Fig. 1. Neighbour-joining tree, based on 16S rRNA gene sequences, showing the phylogenetic relationships of strain Ch07^T and representative members of the family *Alcaligenaceae*. Bootstrap values (>50%) based on 1000 replications are shown as percentages at branching points. Filled circles indicate that the corresponding nodes were also recovered in the tree generated with the maximum-parsimony algorithm. Filled triangles indicate that the corresponding nodes were also recovered in maximum-likelihood tree. Bar, 0.005 substitutions per nucleotide position.

36.5–47.5 mol% (Busse & Stolz, 2006). The DNA G+C content of strain Ch07^T was determined to be 67.9 ± 0.1 mol%.

Strain Ch07^T was found to be a facultatively anaerobic, Gram-negative, motile, short rod-shaped beige-coloured bacterium (1.2–2.1 µm in length and 0.3–0.9 µm in diameter). Cells of the strain produced fimbriae (7–8 nm in width; see Supplementary Fig. S1, available in IJSEM Online) and had three flagella (see Supplementary Fig. S2). Compared with those of *Achromobacter xylosoxidans* subsp. *xylosoxidans* DSM 10346^T and *Bordetella pertussis* DSM 5571^T, the flagella of strain Ch07^T and *Pusillimonas noertemannii* BN9^T were easily lost during sample preparation for microscope observation. Strain Ch07^T and *Achromobacter xylosoxidans* subsp. *xylosoxidans* DSM 10346^T were able to grow under anaerobic conditions, whereas other closely related strains such as *Pusillimonas noertemannii* BN9^T and *Bordetella pertussis* DSM 5571^T were unable to grow anaerobically. The results of comparisons of differential characteristics between strain

Ch07^T and related type strains are summarized in Table 1 and Table 2.

The cellular fatty acid profiles of strain Ch07^T and related strains are shown in Table 2. When strain Ch07^T was cultivated on TSA, the major cellular fatty acids were similar to those found when the strain was cultivated on NA. The affiliation of strain Ch07^T to the family *Alcaligenaceae* was unambiguously demonstrated by 16S rRNA gene sequence comparisons and phylogenetic analyses. However, the fatty acid profile of strain Ch07^T [absence of hydroxy fatty acids and presence of summed feature 5 ($C_{18:1\omega7c/\omega9t/\omega12t}$)] easily differentiated the novel strain from *Pusillimonas noertemannii* BN9^T. The growth of *Pusillimonas noertemannii* BN9^T and *Bordetella pertussis* DSM 5571^T was slightly slower on NA when compared with that of strain Ch07^T and *Achromobacter xylosoxidans* subsp. *xylosoxidans* DSM 10346^T. The composition of fatty acids in *Pusillimonas noertemannii* BN9^T determined in this study was different from those previously reported by Stolz *et al.* (2005). The report of

Table 1. Differential phenotypic characteristics between strain Ch07^T and related type strains

Strains: 1, *Parapusillimonas granuli* Ch07^T; 2, *Pusillimonas noertemannii* BN9^T; 3, *Achromobacter xylosoxidans* sub. *xylosoxidans* DSM 10346^T; 4, *Bordetella pertussis* DSM 5571^T. All the strains had Q8 as the major isoprenoid quinone, showed positive activities for urease and esterase (C4) and assimilated D-glucose, malate, citrate, propionic acid, L-proline, L-serine and valeric acid. All strains gave a negative result in tests for the following: indole production, reduction of nitrates to nitrites and activities of α -fucosidase, α -mannosidase, *N*-acetyl- β -glucosaminidase, α -glucosidase, β -glucosidase, β -glucuronidase, β -galactosidase, α -chymotrypsin, trypsin and lipase (C14). Assimilation of the following is negative for all strains: L-arabinose, L-fucose, melibiose, salicin, D-mannitol, maltose, mannose, arabinose, sucrose, inositol, D-ribose, L-rhamnose, glycogen, potassium 5-ketogluconate, sodium malonate, *N*-acetyl-D-glucosamine, *p*-nitrophenyl- β -D-galactopyranoside and gelatin. All strains are weakly positive for valine arylamidase. +, Positive; –, negative; w, weakly positive. All data are from this study.

Characteristic	1	2	3	4
Cell size (μm)	0.9×1.5	0.7×1.1	0.7×1.5	0.8×1.4
Flagella (number)	3	2	1	1
H ₂ S production	–	–	+	–
Growth in anaerobic conditions	+	–	+	–
Enzyme activities				
Acid phosphatase	–	–	+	–
Alkaline phosphatase	–	–	w	–
Arginine dihydrolase	+	w	+	+
Cystine arylamidase	–	–	–	–
α -Galactosidase	–	–	w	–
Leucine arylamidase	+	–	w	w
Lipase (C8)	+	–	–	w
Naphthol-AS-BI-phosphohydrolase	–	w	+	w
Acidification of glucose	+	–	+	–
Assimilation of				
Adipate	+	–	+	–
L-Alanine	+	–	w	–
Caprate	–	–	+	–
Gluconate	+	–	+	w
L-Histidine	w	w	–	+
3-Hydroxybenzoic acid	+	+	–	+
4-Hydroxybenzoic acid	+	+	–	+
3-Hydroxybutyric acid	+	+	–	+
Itaconic acid	w	+	–	–
Lactic acid	+	+	w	–
Phenylacetate	+	–	+	–
Potassium 2-ketogluconate	+	–	–	w
Sodium acetate	+	+	w	–
D-Sorbitol	+	–	–	–
Suberic acid	–	+	+	+
DNA G + C content (mol%)*	67.9	61.8 ^a	69.2 ^b	63.8 ^c

*Data from: *a*, Stolz *et al.* (2005); *b*, Yabuuchi & Yano (1981) and *c*, Vancanneyt *et al.* (1995).

Stolz *et al.* (2005) showed *Pusillimonas noertemannii* BN9^T contained large amounts of C_{16:0} (19%), C_{17:0} cyclo (31%) and C_{19:0} cyclo 8c (27%). However, our results showed the following fatty acid content: C_{16:0} (19.7%), C_{17:0} cyclo (18.8%), summed feature 3 (C_{16:1} ω 7c/C_{15:0} iso 2-OH; 5.3%) and summed feature 5 (C_{18:1} ω 7c/ ω 9t/ ω 12t) was not detected. The quantitative differences in fatty acid contents found between this study and previous studies may have been due to the use of biomass harvested

from different incubation conditions and at different growth phases.

The predominant quinone of strain Ch07^T was Q-8. This quinone system supported our assignment of strain Ch07^T to the order *Burkholderiales* as most of the species in this order also exhibit Q-8 as the predominant quinone. Strain Ch07^T possessed polar lipids such as phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and diphosphati-

Table 2. Cellular fatty acid composition (%) of strain Ch07^T and related type strains

Strains: 1, *Parapusillimonas granuli* Ch07^T; 2, *Pusillimonas noertemanni* BN9^T; 3, *Achromobacter xylosoxidans* subsp. *xylosoxidans* DSM 10346^T; 4, *Bordetella pertussis* DSM 5571^T. All strains were grown on TSA at 30 °C for 48 h prior to fatty acid analysis. For unsaturated fatty acids, the position of the double bond is located by counting from the methyl (ω) end of the carbon chain. The *cis* and *trans* isomers are indicated by the suffixes *c* and *t*, respectively. ND, not detected; tr, trace amount <0.5 %.

Fatty acid	1	2	3	4
Saturated				
C _{12:0}	ND	5.9	1.4	3.8
C _{14:0}	ND	2.7	2.1	tr
C _{16:0}	33.2	19.7	37.2	14.8
C _{17:0} cyclo	18.2	18.8	8.3	16.9
C _{18:0}	tr	2.4	2.7	4.7
C _{19:0} cyclo ω 8 <i>c</i>	ND	13.6	tr	ND
C _{19:0} 10 methyl TBSA	ND	ND	ND	9.9
Unsaturated				
C _{14:1} ω 5 <i>c</i>	ND	1.2	ND	ND
C _{18:1} ω 7 <i>c</i>	ND	5.0	8.9	2.9
C _{18:1} ω 9 <i>c</i>	ND	2.8	tr	6.5
C _{19:0} cyclo ω 8 <i>c</i>	0.9	ND	tr	15.8
C _{20:2} ω 6, 9 <i>c</i>	ND	0.9	ND	ND
Hydroxy				
C _{12:0} 2-OH	ND	3.3	1.6	2.6
C _{14:0} 2-OH	ND	ND	ND	1.8
C _{16:0} 3-OH	ND	1.51	tr	1.6
C _{18:1} 2-OH	ND	3.7	ND	2.2
Summed features*				
2	7.4	13.4	9.8	6.8
3	28.5	5.3	25.9	2.9
5	11.3	ND	ND	ND

*Summed features are groups of two or three fatty acids that cannot be separated by GLC with the MIDI system. Summed feature 2 contains C_{16:1} isoI/C_{14:0} 3-OH and/or C_{12:0} alde. Summed feature 3 contains C_{16:1} ω 7*c*/C_{15:0} iso 2-OH. Summed feature 5 contains C_{18:1} ω 7*c*/ ω 9*t*/ ω 12*t*.

dyglycerol (DPG), which are common in members of the genera *Pusillimonas*, *Alcaligenes* and *Castellaniella* of the family *Alcaligenaceae* (see Supplementary Fig. S3, available in IJSEM online). *Pusillimonas noertemanni* BN9^T was found to contain polar lipids PE, DPG and PG and the results from this study were similar to those found by Stolz *et al.* (2005). The major polyamine of the strain Ch07^T was putrescine, which is a common feature of members of the class *Betaproteobacteria*.

On the basis of phylogenetic and chemotaxonomic data, strain Ch07^T was clearly different from strains belonging to the genera *Pusillimonas*, *Achromobacter* and *Bordetella*. It is proposed that the strain Ch07^T represents a novel species of a new genus. The name *Parapusillimonas granuli* gen. nov., sp. nov. is proposed for this new taxon.

Description of *Parapusillimonas* gen. nov.

Parapusillimonas (Pa.ra.pu.sil.li.mo'nas. Gr. prep. *para* like; N.L. fem. n. *Pusillimonas* bacterial genus name; N.L. fem. n. *Parapusillimonas* a bacterium like *Pusillimonas*).

Cells are Gram-reaction-negative. Tests for catalase and oxidase are positive. Ubiquinone Q-8 is present. When incubated on TSA, the major cellular fatty acids are C_{16:0}, C_{17:0} cyclo, summed feature 3 (C_{16:1} ω 7*c*/C_{15:0} iso 2-OH) and summed feature 5 (C_{18:1} ω 7*c*/ ω 9*t*/ ω 12*t*). The major polyamine is putrescine. The polar lipids are PE, PG and DPG. The G+C content of the genomic DNA is high (67.9±0.1 mol%). The type species of the genus is *Parapusillimonas granuli*.

Description of *Parapusillimonas granuli* sp. nov.

Parapusillimonas granuli (gra.nu'li. L. gen. n. *granuli* of a small grain, pertaining to a granule, from which the type strain was isolated).

Facultatively anaerobic, short rods (0.9 µm × 1.5 µm). Cells are motile by means of three flagella per cell. Fimbriae (Φ7.56 nm) are located on the cell surface. When grown on R2A agar at 30 °C for 5 days, colonies of the type strain are beige-coloured and circular and have a diameter of about 2 mm. Grows on R2A agar at 25–37 °C and at pH 6.5–7.5. The optimal growth temperature and pH are 30 °C and pH 7, respectively. Produces arginine dihydrolase, urease, esterase (C4), esterase (C8), leucine arylamidase and valine arylamidase. Gives a negative result in tests for the following: production of H₂S and indole, reduction of nitrates to nitrites and activities of β-glucosidase, α-glucosidase, β-galactosidase, α-galactosidase, β-glucuronidase, alkaline phosphatase, acid phosphatase, protease, cystine arylamidase, naphthol-AS-BI-phosphohydrolase, α-fucosidase, α-mannosidase, N-acetyl-β-glucosaminidase, α-chymotrypsin, trypsin and lipase (C14). Assimilates D-glucose, D-sorbitol, adipate, phenylacetate, sodium acetate, lactic acid, 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, 3-hydroxybutyric acid, potassium 2-ketogluconate, malate, citrate, propionic acid, valeric acid, L-proline, L-alanine, L-serine, L-histidine and gluconate. Does not assimilate L-arabinose, caprate, L-fucose, melibiose, maltose, D-mannitol, sucrose, inositol, D-ribose, L-rhamnose, salicin, glycogen, potassium 5-ketogluconate, sodium malonate or suberic acid. The predominant quinone is Q-8.

The type strain, Ch07^T (=KCTC 12668^T=LMG 24012^T), was isolated from brownish-black granules from an upflow anaerobic sludge blanket reactor used to treat brewery wastewater, Daejeon, Republic of Korea. The DNA G+C content of the type strain is 67.9±0.1 mol%, as determined by HPLC.

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