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ORIGINAL PAPER

Pontibacter chitinilyticus sp. nov., a novel chitin-hydrolysing bacterium isolated from soil

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Abstract A Gram-stain negative, non-motile, orange-red-pigmented, asporogenous, rod-shaped bacterial strain, designated 17gy-14^T, was isolated from a soil sample collected from a Seoul Women's University field. The strain can grow at 7–37 °C, pH 6.0–8.0, and can tolerate up to 5.5% (w/v) NaCl concentration. Flexirubin-type pigments were absent. Phylogenetic analysis based on 16S rRNA gene sequences indicated that the strain 17gy-14^T strain belongs to the genus *Pontibacter* in the family *Cytophagaceae* with sequence similarities ranging from 95.4 to 92.9% with other type strains of the genus *Pontibacter*. The predominant cellular fatty acids were identified as iso-$C_{15:0}$, anteiso-$C_{15:0}$, iso-$C_{17:0}$ 3-OH and summed feature 4 (comprising iso-$C_{17:1}$ I/anteiso-$C_{17:1}$ B). The predominant menaquinone was identified as MK-7. The DNA G+C content was determined to be 48.7 mol%. The major polar lipid was found to be phosphatidylethanolamine. Based on phenotypic, chemotaxonomic and phylogenetic analyses, strain 17gy-14^T is concluded to represent a novel species of the genus *Pontibacter* in the phylum *Bacteroidetes*, for which the name *Pontibacter chitinilyticus* sp. nov. is proposed. The type strain is 17gy-14^T (=KCTC 52914^T =NRBC 113056^T).

Keywords *Pontibacter chitinilyticus* sp. nov · Chitin hydrolysis · Polyphasic taxonomy · *Cytophagaceae* · Soil bacteria

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Introduction

The genus *Pontibacter* was first proposed by Nedashkovskaya et al. (2005) with *Pontibacter actiniarum* as the type species. Later the description of this genus was emended by Wang et al. (2010). Currently, the genus *Pontibacter* comprises validly named 30 species, which have been isolated from various environments such as actinians (Nedashkovskaya et al. 2005), desert (Zhang et al. 2008), soil (Cao et al. 2014), seawater (Kang et al. 2013), tidal flats (Park et al. 2016), rhizospheric soil (Xu et al. 2014), soil of a solar saltern (Joung et al. 2011, 2013; Subhash et al. 2013) and hexachlorocyclohexane-contaminated soil (Singh et al. 2017). *Pontibacter diazotrophicus* has been reported as the only nitrogen-fixing species in this genus to date (Xu et al. 2014). Members of this genus are aerobic, Gram-stain negative, rod-shaped,

asporogenous, motile or non-motile by gliding. They possess phosphatidylethanolamine as their major polar lipid and menaquinone-7 (MK-7) as the major or sole respiratory quinone. The DNA G+C content of members of the genus is in the range 34–65 mol%. The aim of this study was to determine the taxonomic position of a new soil isolate, designated 17gy-14^T, by using polyphasic taxonomic characterisation. Strain 17gy-14^T was isolated during a study of bacterial diversity of field soil near the Seoul Women's University, South Korea. This study describes the strain as a novel member of the genus *Pontibacter*.

Materials and methods

Isolation of the bacterial strain and culture conditions

Strain 17gy-14^T was isolated from soil near the Seoul Women's University, South Korea (GPS: N 37′37.445″ E 127′ 5.22.8″). For isolation, 5 g of soil was suspended in 10 ml of sterile saline (0.85% NaCl, w/v). The serial dilutions were spread onto R2A agar (Difco). Several types of colonies were observed after one week of incubation at an ambient temperature (20–25 °C). Among them, an orange-red-coloured colony was selected. Pure colonies of strain 17gy-14^T were obtained after repeatedly streaking the bacterial culture on R2A medium. The pure culture of strain 17gy-14^T was stored at 4 °C for temporary maintenance and sub-cultured every 2 weeks. Based on 16S rRNA gene sequence similarities and phylogenetic analyses, *Pontibacter xinjiangensis* NBRC 107674^T and *Pontibacter korlensis* KACC 15371^T were selected for comparative analyses, obtained from NITE Biological Resource Center (NBRC) and Korean Agriculture Culture Collection (KACC) and were used as reference strains. In addition the type species of the genus, *P. actiniarum* KACC 15405^T, obtained from Korean Agriculture Culture Collection (KACC), was also selected for comparative physiological and chemotaxonomic analyses. Strain 17gy-14^T and the reference strains were routinely sub-cultured on R2A agar at 30 °C and maintained in R2A broth (Difco) supplemented with 50% (v/v) glycerol at − 80 °C.

Morphological, physiological and biochemical analyses

Cell morphology was observed by transmission electron microscopy (TEM) (LIBRA120, Carl Zeiss, Germany) using cells from exponentially growing cultures and motility was assessed in R2A medium containing 0.4% agar. Gliding motility was tested using the hanging drop method after growing the cells in R2A broth (Difco) for 48 h at 30 °C (Bernardet et al. 2002). Gram-staining was determined by the non-staining KOH lysis method (3% KOH; Buck 1982). The production of flexirubin-type pigment was investigated by flooding with 20% (w/v) KOH solution on agar plates (Fautz and Reichenbach 1980). Catalase activity was determined by the production of bubbles with 3% (v/v) hydrogen peroxide (H_2O_2). Oxidase activity was determined using 1% (w/v) tetra-methyl-p-phenylenediamine dihydrochloride. Growth of strain 17gy-14^T was assessed at 30 °C for 7 days on several bacteriological agar media: R2A agar (MB cell), Marine agar (Difco), Tryptic soy agar (Difco), Luria–Bertani agar (Difco), and Nutrient agar (Difco). The temperature range for growth was determined on R2A agar 4, 10, 20, 28, 30, 37, 40 and 42 °C by observing the formation of colonies. Growth in NaCl was examined by cultivation in R2A broth containing 0–6% NaCl (w/v, at 0.5% intervals). Growth under anaerobic conditions was observed via incubation in a GasPak jar (BBL, Cockeysville, MD, USA) at 30 °C for 7 days. The pH range for growth was determined by cultivation at 30 °C in R2A broth adjusted to pH 4–10 (at pH 1 unit intervals) before sterilization with citrate/NaH_2PO_4 buffer (pH 4.0–5.0), phosphate buffer (pH 6.0–8.0) and Tris buffer (pH 9.0–10.0) (Breznak and Costilow 2007). Hydrolysis of Tween 40, Tween 60 and Tween 80 was examined as described by Smibert and Krieg (1994). Hydrolysis of chitin, starch, CM-cellulose and casein was evaluated as described previously (Dahal and Kim 2017). Other biochemical properties and enzyme activities were tested using API ZYM and API 20NE kits (bioMérieux) following the manufacturer's instructions.

Sequencing and phylogenetic analysis of 16S rRNA gene

The genomic DNA of the strain was extracted using cetyl trymethylammonium bromide/NaCl solution

according to the standard protocol. The 16S rRNA gene sequence of strain 17gy-14^T was amplified using the universal bacterial primer set 27F, 518F, 800R and 1492R (Weisburg et al. 1991). The 16S rRNA gene sequence of strain 17gy-14^T (1487 bp) was compared with 16S rRNA gene sequences of closely related taxa obtained from GenBank. This comparison was done using the Seqman software (DNASTAR Inc., Madison, WI, USA) and the EzTaxon-e service (Kim et al. 2012); the sequences were edited using the BioEdit program. Multiple sequences were aligned using MEGA 7 software (Kumar et al. 2016) and analysed using CLUSTALX 2.1 (Thompson et al. 1997). Phylogenetic trees were constructed according to the neighbour-joining (Felsenstein 1985), maximum-parsimony and maximum-likelihood methods with the Kimura two-parameter model (Kimura 1980). A minimum-evolution tree was also constructed using the MEGA 7 software to estimate the confidence of tree topologies (Rzhetsky and Nei 1992). During phylogenetic analysis, evolutionary distances were calculated using the Kimura two-parameter model (Kimura 1980) and bootstrap values were calculated based on 1000 replications (Felsenstein 1985).

Chemotaxonomic characterisation

The respiratory quinone was extracted with chloroform/methanol (2:1, v/v), evaporated under a vacuum, re-extracted with acetone and analysed using high-performance lipid chromatography (HPLC) according to previous reports (Hiraishi et al. 1996; Collins and Jones 1981). For the fatty acid analysis, cellular biomass of the strain 17gy-14^T and reference strains were harvested from R2A plates incubated at 30 °C for 4 days. Cellular fatty acids were obtained by saponification, methylation and extraction, as reported previously (Kuykendall et al. 1988). The Sherlock Microbial Identification System V6.01 (MIS, database TSBA6, MIDI Inc., Newark, DE, USA) was used to identify the extracted fatty acids.

Polar lipids were extracted by the method described by Minnikin et al. (1984). The polar lipids were analysed by two-dimensional TLC using chloroform/methanol/water (65:25:4; v/v/v) in the first dimension and chloroform/methanol/acetic acid/water (80:15:12:4; v/v/v/v) in the second. Appropriate detection reagents (Komagata and Suzuki 1987) were used to identify the spots; phosphomolybdic acid (Sigma), molybdenum blue spray reagent (Sigma), α-napthol and ninhydrin on the plates to identify the spots.

Determination of DNA base composition

The measurement of the G+C content of the chromosomal DNA for strain 17gy-14^T was determined by a simple fluorimetric method using SYBR Green 1 (Life Technologies, Waltham, USA) and a real-time PCR thermocycler (Rotor-Gene Q, Qiagen, Hilden, Germany). Genomic DNA of *Lactococcus lactis subsp. lactis* KACC 13877^T, *Bacillus subtilis subsp. subtilis* KACC 17796^T, *Bacillus licheniformis* KACC 10476^T, *Escherichia coli* KACC 14818^T, *Corynebacterium glutamicum* KACC 20786^T, *Pseudomonas aeruginosa* ATCC 15442^T and *Micrococcus luteus* KACC 13377^T were used as references for calibration (Ausubel et al. 1995).

Results and discussion

Phylogenetic analysis and DNA G+C content

The nearly complete 16S rRNA gene sequence of strain 17gy-14^T comprises a continuous length of 1487 bp and has been deposited in Genbank (accession number KY924850). Based on the 16S rRNA gene sequence similarity, the close relatives of this strain were identified as *P. xinjiangensis* 311-10^T (95.4%), *P. korlensis* X14-1^T (95.2%) and *P. actiniarum* KMM6156^T (94.8%). These data suggested that strain 17gy-14^T should not be assigned to any recognised *Pontibacter* species and should be considered to represent a novel species within this genus. Phylogenetic analysis based on the neighbor-joining phylogenetic tree (Fig. 1) indicated that strain 17gy-14^T clustered with members of the genus *Pontibacter*. The placement of the strain within the genus *Pontibacter* was also supported by the tree created by the maximum-likelihood algorithm (Supplementary Fig S1).

The G+C content of the genomic DNA of strain 17gy-14^T was determined to be 48.7 mol%, a value within the range reported for members of the genus *Pontibacter* (Table 1).

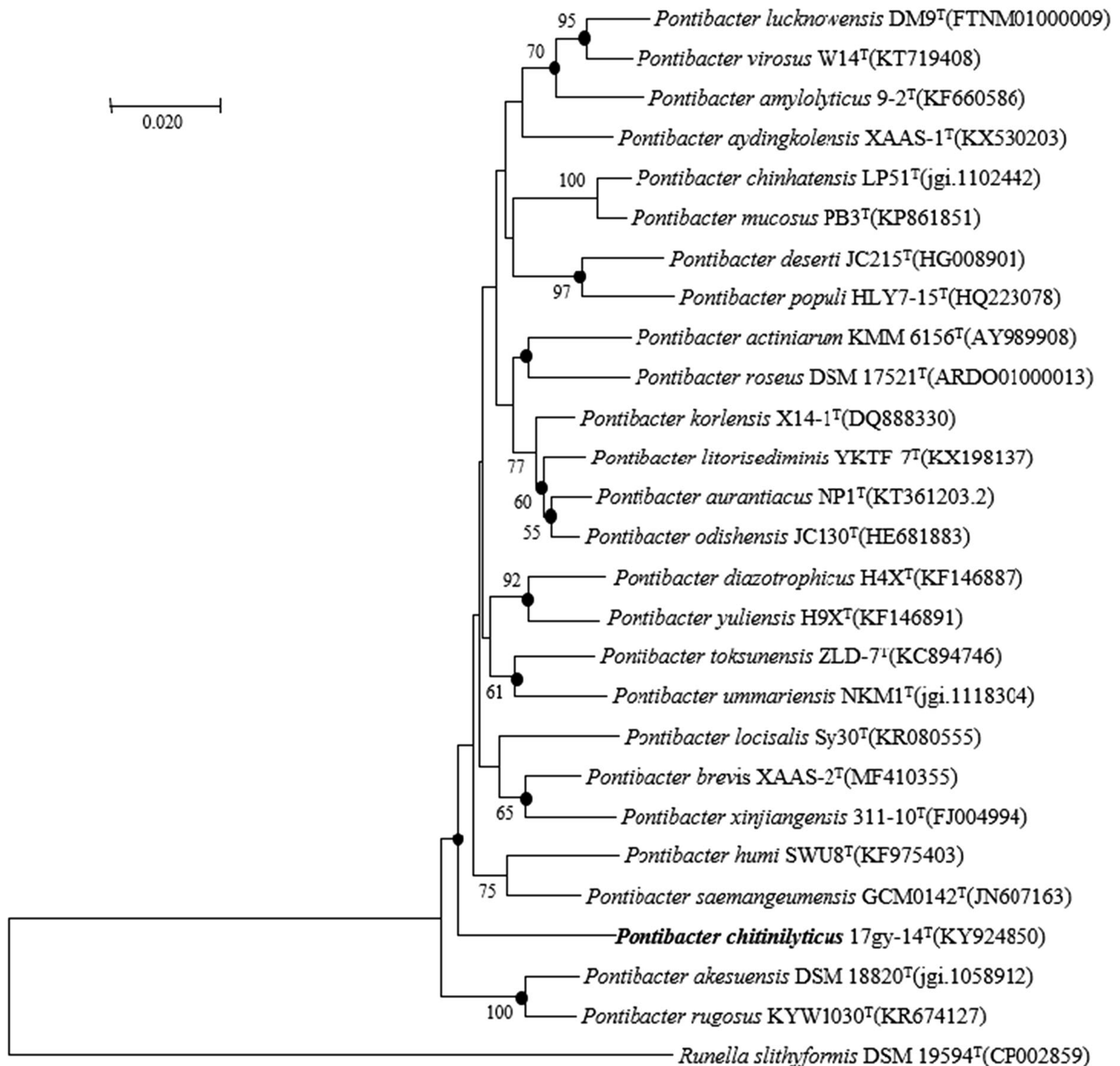

Fig. 1 Neighbour-joining phylogenetic tree showing the relationship of strain 17gy-14^{T} to members of the genus *Pontibacter*, based on 16S rRNA gene sequences analysis. Percentages at nodes are levels of bootstrap support (> 50%) from 1000 resampled datasets. Filled circles indicate nodes recovered by all three treeing methods (neighbor-joining, maximum-likelihood, and maximum-parsimony). *Runella slithyformis* DSM 19594^{T} (CP002859) was used as an out-group. GenBank accession numbers are given in parentheses. Bar, 0.020 substitutions per nucleotide position

Phenotypic characteristics

Strain 17gy-14^{T} was found to be aerobic, non-motile, asporogenous and Gram-stain negative. Cells were observed to be rod-shaped, 0.6–0.8 μm wide and 1.0–1.7 μm long (Supplementary Fig. 2). Strain 17gy-14^{T} was found to show the following enzyme activities: positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine acrylamidase, valine acrylamidase, cystine acrylamidase, trypsin, α-chymotrypsin, acid phosphatase, naphtol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase; and negative for, lipase (C14) and β-glucuronidase.

Table 1 Phenotypic characteristics that differentiate strain 17gy-14[T] from phylogenetically closely related type strains of the genus *Pontibacter*

Characteristic	1	2	3	4
Isolation source	Soil	Soil	Sand	Actinians
Colony color	Orange-red	Pink	Pink	Pink
Temperature range (°C)	6–37	4–37	7–45	6–43
Oxidase	+	−	+	+
Gliding motility	−	−	+	+
Salinity range (%) for growth	0–5.5	0–5	0–8	0–10
Hydrolysis of				
DNA	−	−	+	+
Starch	+	+	+	−
Chitin	+	+	+	−
Casein	−	+	−	−
Tween 80	+	−	−	−
Gelatin	−	−	+	+
β-galactosidase	+	−	+	+
Enzyme activities (API ZYM)				
Cystine arylamidase	+	+	−	−
α-galactosidase	+	−	+	−
α-glucosidase	+	−	+	+
α-fucosidase	+	−	−	−
Assimilation of (20NE)				
D-glucose	+	−	+	−
L-arabinose	+	+	−	−
D-mannose	+	−	+	+
D-mannitol	+	−	−	+
N-acetyl-D-glucosamine	+	−	+	+
D-maltose	+	−	+	−
Potassium gluconate	+	+	−	−
Capric acid	−	−	−	−
Adipic acid	−	+	−	−
Malic acid	+	+	−	−
DNA G+C content (mol%)	47.8	(47.8)	(48.2–48.9)	(48.7)

Strains: 1, 17gy-14[T]; 2, *Pontibacter xinjiangensis* NBRC 107674[T] (Wang et al. 2010); *Pontibacter korlensis* KACC 15371[T] (Zhang et al. 2008) and *Pontibater actiniarum* KACC 15405[T] (Nedashkovskaya et al. 2005). All data are from the present study except those indicated in parentheses, which were taken from the literature (Wang et al. 2010; Zhang et al. 2008; Nedashkovskaya et al. 2005)

+positive, −negative

The following substrates are assimilated: D-glucose, L-arabinose, D-mannose, D-mannitol, *N*-acetyl-glucoseamine, D-maltose, potassium gluconate and malic acid.

Chemotaxonomic characteristics

The predominant respiratory quinone in strain 17gy-14[T] was found to be menaquinone-7 (MK-7), which is consistent with that described for the genus *Pontibacter*. The major cellular fatty acids identified were iso-$C_{15:0}$ (20.9%), anteiso-$C_{15:0}$ (11.1%), iso-$C_{17:0}$ 3OH (8.5%) and summed feature 4 (comprising iso-$C_{17:1}$ I/anteiso-$C_{17:1}$ B), (26.2%, as defined by the MIDI system). A comparison of the fatty acid profile of strain 17gy-14[T] and related type strains is shown in Table 2. Some qualitative and quantitative differences in fatty acid contents were observed between strain 17gy-14[T] and its close relatives *P. xinjiangensis* 311-10[T], *P. korlensis* X14-1[T] and *P. actiniarum* KMM6156[T]. The presence of $C_{14:1}\omega5c$ (5.0%), anteiso-$C_{17:0}$ (3.6%), iso-$C_{17:0}$ 2-OH (1.7%) and the absence of iso-$C_{18:0}$, anteiso-$C_{16:0}$, summed features 5 and 8 in strain 17gy-14[T] distinguished it from the related type strains. In addition, the amount of summed feature 3 in strain 17gy-14[T] (comprising

Table 2 Cellular fatty acid profiles of strain 17gy-14^T and related type strains in the genus *Pontibacter*

Fatty acid	1	2	3	4
Saturated				
$C_{14:0}$	1	TR	TR	1.1
$C_{16:0}$	TR	2.4	TR	2.3
$C_{17:0}$	1.5	TR	1.5	1.6
$C_{18:0}$	–	2.9	1	1.2
Unsaturated				
$C_{17:1}$ ω7c	1.6	–	2	–
$C_{17:1}$ ω6c	2.1	4.3	2.3	1.1
$C_{17:1}$ ω5c	1.2	1.5	1.6	4.3
$C_{17:1}$ iso ω5c	5.5	–	–	TR
$C_{14:1}$ ω5c	5	–	6.4	–
$C_{16:1}$ ω5c	2.2	6.9	4.7	TR
$C_{19:0}$ cyclo ω8c	1.7	–	2.1	–
Branched				
$C_{15:1}$ iso F	1.8	TR	1.7	TR
iso-$C_{15:0}$	20.9	16.2	15.9	22.8
anteiso-$C_{15:0}$	11.1	1.5	3.1	2.7
$C_{16:1}$ iso H	2.6	–	1.9	TR
anteiso-$C_{16:0}$	–	TR	3.7	–
iso-$C_{17:0}$	4.5	1.8	5.9	5.7
anteiso-$C_{17:0}$	3.6	TR	TR	TR
iso-$C_{19:0}$	1.8	–	2.7	–
Hydroxylated				
$C_{16:1}$ iso H	2.6	–	1.9	TR
iso-$C_{15:0}$ 3-OH	2.5	2.8	3.3	3.3
$C_{17:0}$ iso 3-OH	8.5	7.6	7.5	8.6
$C_{17:0}$ 2-OH	1.7	–	–	–
Summed features[a]				
3	5.3	14.5	11.3	12.4
4	26.2	25.7	13.9	29.8
5	–	1.3	TR	1.5
8	–	1.3	TR	1.3

Strains: 1, 17gy-14^T; 2, *Pontibacter xinjiangensis* NBRC 107674^T; *Pontibacter korlensis* KACC 15371^T and *Pontibater actiniarum* KACC 15405^T. All data were obtained in this study. Values are percentages of total fatty acids, and only fatty acids representing more than 1% for at least one of the strains are shown; – not detected, TR trace amounts (< 1%)

[a]Summed features represent groups of two or three fatty acids that cannot be separated using MIDI system. Summed features consist of: 3, $C_{16:1}\omega$7c and/or $C_{16:1}\omega$6c; 4, iso-$C_{17:1}$ I/anteiso-$C_{17:1}$ B; 5, anteiso-$C_{18:0}$ and/or $C_{18:2}$ ω6,9c; 8, $C_{18:1}\omega$7c and/or $C_{18:1}\omega$6c

$C_{16:1}\omega$7c and/or $C_{16:1}\omega$6c) was only 5.3% which was lower than the amount in the closely related type strains. The polar lipid profile of the strain 17gy-14^T was found to contain phosphatidylethanolamine as the diagnostic component (Supplementary Fig. 2). In addition, four unidentified glycolipids, an unidentified aminophosphoglycolipid, two unidentified phosphoglycolipids, two phospholipids and three unidentified polar lipids were also detected. The polar lipid profile distinguished the strain 17gy-14^T from its close relatives supporting the conclusion that it represents a novel species.

Taxonomic conclusion

The predominant menaquinone of the strain 17gy-14^T was MK-7, the same as for other *Pontibacter* species. The strain 17gy-14^T contained phosphatidylethanolamine as the major phospholipid and the major fatty acids of the strain 17gy-14^T were anteiso-C15:0, iso-C15:0, iso-$C_{17:0}$ 3-OH and summed feature 4 (comprising iso-$C_{17:1}$ I/anteiso-$C_{17:1}$ B). These chemotaxonomic data suggest that strain 17gy-14^T represents a novel species of the genus *Pontibacter*. In addition, based on its phylogenetic distance from established *Pontibacter* species and the combination of phenotypic characteristics (Table 1), it is clear that strain 17gy-14^T is not affiliated with any recognised species within the genus. Therefore, according to the data presented, strain 17gy-14^T should be placed in the genus *Pontibacter* as representative of novel species, for which the name *Pontibacter chitinilyticus* sp. nov. is proposed. The Digital Protologue database TaxonNumber for strain 17gy-14^T is TA00770.

Description of *Pontibacter chitinilyticus* sp. nov.

Pontibacter chitinilyticus sp. nov. (chi.ti.ni.ly'ticus. N.L. n. *chitinum*, chitin; N.L. suff.-*lyticus*, dissolving; N.L. masc. adj. *chitinilyticus*, chitin-dissolving).

Cells are aerobic, non-motile, asporogenous, Gram-stain negative, catalase and oxidase positive, rod-shaped, 0.6–0.8 µm wide and 1.0–1.7 µm long. Does not require Na^+ or sea water for growth. Colonies on R2A agar are red–orange, circular, 2.3 mm in diameter, convex, shiny and smooth. Grows well on R2A and Marine agar, grows weakly on Tryptic soy agar and nutrient agar; does not grow on LB agar. Grows at

temperatures 7–37 °C (optimum, 28–30 °C), pH 6.0–8.0 (optimum, 6.5–7.5), and optimally in the absence of NaCl but can tolerate up to 5.5% NaCl. No growth observed at 6 and 38 °C. Flexirubin-type pigments are absent. Positive for hydrolysis of starch, Tween 20 and 80 and chitin but negative for hydrolysis of DNA, casein and CM cellulose. Nitrate is not reduced to nitrite. Glucose is not fermented. Positive for esculin ferric citrate, arginine dihydrolase and urease tests. Gelatin is not liquefied. Indole test is negative. The quinone system contains significant amounts of MK-7 and the major fatty acids are iso-$C_{15:0}$, anteiso-$C_{15:0}$, iso-$C_{17:0}$ 3OH and summed feature 4 (comprising iso-$C_{17:1}$ I/anteiso-$C_{17:1}$ B). The major polar lipid is phosphatidylethanolamine. The genomic DNA G+C content of the type strain is 48.7 mol%.

The type strain 17gy-14^{T} (=KCTC 52914^{T}=NBRC 113056^{T}) was isolated from soil near Seoul Women's University, South Korea. The GenBank accession number for the 16S rRNA gene sequences of strain 17gy-14^{T} is KY924850.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

Ethical standard This study does not describe any experimental work related to human.

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