Pectinatus brassicae sp. nov., a Gram-negative, anaerobic bacterium isolated from salty wastewater

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A novel Gram-negative, non-spore-forming, strictly anaerobic, heterotrophic bacterium, strain TY^T, was isolated from salty pickle wastewater. Cells were rod-shaped with comb-like flagella, slightly curved and very variable in length. Optimal growth occurred at 28 °C and pH 6.5. Cells were resistant to up to 50 g NaCl l^{-1}. Strain TY^T produced acid from glycerol, sucrose, glucose, fructose and mannitol. The main fermentation products from glucose were acetic and propionic acids. Tests for acid phosphatase and naphthol-AS-BI-phosphohydrolase activities were positive. The major fatty acids were $C_{14:0}$ DMA (18.7 %), $C_{15:0}$ (15.4 %), anteiso-$C_{18:1}$ (15.2 %), $C_{11:0}$ (13.3 %) and summed feature 5 ($C_{17:1}\omega7c$ and/or $C_{17:2}$) (11.0 %). The DNA G+C content was 35.9 mol%. 16S rRNA gene sequence-based phylogenetic analysis indicated that strain TY^T represented a novel species of the genus *Pectinatus* (sequence similarity to other members of the genus ranged from 93.2 to 94.8 %). Based on its phenotypic, genotypic and phylogenetic characteristics, strain TY^T is proposed to represent a novel species, named *Pectinatus brassicae* sp. nov. (type strain TY^T =JCM 17499^T =DSM 24661^T).

The genus *Pectinatus* is composed of several Gram-negative, rod-shaped, strictly anaerobic and flagellated bacteria (Lee *et al.*, 1978; Schleifer *et al.*, 1990; Gonzalez *et al.*, 2004; Juvonen & Suihko, 2006). At the time of writing, the names of four species, *Pectinatus cerevisiiphilus* (the type species), *P. frisingensis*, *P. haikarae* and *P. portalensis*, have been validly published. According to Vereecke & Arahal (2008), however, the name *Pectinatus portalensis* should be rejected because the cultures cited as the type strain do not conform to the original description of the species, and the type strain is therefore not available from any public collection. Identified *Pectinatus* strains have been isolated from man-made environments only, particularly environments related to beer fermentation (Lee *et al.*, 1978; Schleifer *et al.*, 1990; Juvonen & Suihko, 2006). Here, we describe a novel isolate, TY^T, which, to our knowledge, is the first *Pectinatus* strain to be isolated from pickle wastewater and is proposed to represent a novel species of *Pectinatus*.

Several litres of pickle wastewater (chemical oxygen demand 8500 mg l^{-1}, salinity 3.7 %, pH 4.2, temperature ~25 °C) were collected from Cixi Pickle Mill in Zhejiang province, China, in October 2009. The autoclaved pickle wastewater was used as a solid medium by adding 2 % agar for initial isolation and cultivation of strain TY^T. Medium PYG, containing (per litre distilled water) 10 g peptone, 10 g yeast extract, 5 g glucose, 0.5 g cysteine, 1 mg resazurin and 40 ml salt solution (see DSMZ medium 104), was used for further research and identification. Medium YG (as medium PYG, except that the peptone was replaced by 5 g NH_4Cl l^{-1} and yeast extract was reduced to 1 g l^{-1}) was used to determine the fermentation products from glucose. Hungate roll-tube technique (Hungate, 1969) and an anaerobic chamber (Bugbox; Ruskinn) were used throughout our studies; pure N_2 was used to create anaerobic conditions. *P. cerevisiiphilus* DSM 20467^T, *P. haikarae* DSM 16980^T and *P. frisingensis* DSM 6306^T were purchased from the DSMZ for comparative studies. Unless otherwise stated, cultures were grown in medium PYG at 28 °C.

Cell morphology was examined by optical microscopy (BX 40; Olympus) and electron microscopy (JEM-1230; JEOL) in the stationary phase (incubated for 24 h). Cells of all growth phases were used for Gram staining, while cells from late-exponential and stationary phases were used for spore staining and poly-β-hydroxybutyrate staining, respectively. The rod-shaped cells of strain TY^T showed flagella emanating from one side (Fig. 1), a distinguishing characteristic of the genus *Pectinatus*. Strain TY^T has a cell-wall structure typical of Gram-negative bacteria, which is consistent with the results of Gram staining. In the late-exponential and stationary phases, cells of strain TY^T, *P.*

Abbreviations: DMA, dimethyl acetal; FAME, fatty acid methyl ester.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain TY^T is HM212531.

Two supplementary tables and two supplementary figures are available with the online version of this paper.

cerevisiiphilus DSM 20467^T, *P. haikarae* DSM 16980^T and *P. frisingensis* DSM 6306^T were quite variable in length and contained inclusion granules which were not stained by Sudan black.

To test the pH range for growth, medium PYG was adjusted to pH 3.0–9.0 at intervals of 0.5 pH units; MES (pH 5.5–6.0), PIPES (pH 6.5–7.0), Tricine (pH 7.5–8.0) and CAPSO (pH 8.5–9.0) were added at 25 mM to maintain the pH. The temperature range for growth was determined by incubation at 4, 10, 15, 20, 25, 28, 30, 35, 37, 40, 45 and 50 °C. Medium PYG with NaCl concentrations of 0, 1, 2, 3, 5, 7 and 10 % (w/v) was used to test the salt tolerance. All of these incubations were conducted for 7 days. Acid production tests were performed with Hungate tubes containing medium 50CHB/E (bioMérieux) supplied with the substrates tested in the API 50CH strip (bioMérieux). Use of milk and gelatin was tested in sugar-free medium YG supplied with the two substrates. Active enzymes were detected by using the API ZYM kit, with incubation in anaerobic bags (GENbag anaer; bioMérieux) at 28 °C for 16 and 4 h. NaCl solution (0.85 %, w/v) was prepared for API ZYM (bioMérieux) according to the manufacturer's instructions. Antibiotic sensitivity was tested by using filter paper discs containing penicillin (10 IU), ampicillin (10 μg), chloromycetin (30 μg), neomycin (30 μg), streptomycin (10 μg), kanamycin (30 μg), amoxicillin (10 μg), erythromycin (15 μg) or vancomycin (5 μg). Catalase and oxidase activities were tested as described by Wu *et al.* (2010). The methyl red and Voges–Proskauer tests were performed as described by Lányí (1987). Production of H_2S and indole and reduction of nitrate were examined as described by Dong & Cai (2001). Methanol, ethanol, 1-propanol, 2-propanol, butanol, formic acid, acetic acid, propionic acid, butyric acid, lactate and pyruvic acid were tested as probable fermentation products by HPLC analysis as described by Rajakylä (1981). All of the physiological experiments were performed in duplicate.

Detailed physiological characteristics are summarized in Table 1. Strain TY^T was more tolerant towards salt than the other strains, but failed to grow at 45 °C, a temperature at which *P. cerevisiiphilus* DSM 20467^T could grow. The API tests showed that TY^T and the reference *Pectinatus* type strains had different abilities to produce acid from glycerol,

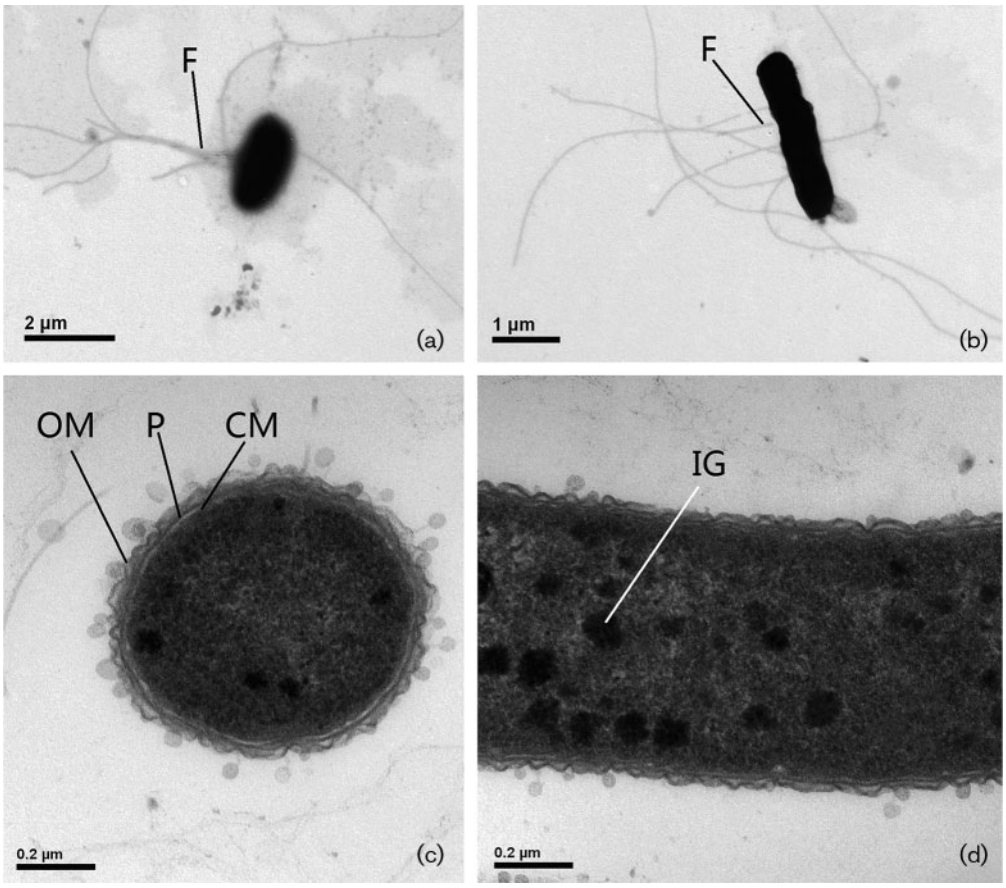

Fig. 1. Micrographs of cells of strain TY^T. Typical Gram-negative cell wall, comb-like flagella and inclusion granules are shown. (a) End view; (b) side view; (c) cross section; (d) longitudinal section. F, Flagella; IG, inclusion granules; CM, cell membrane; OM, outer membrane; P, peptidoglycan.

lactose, D-xylose, mannose, mannitol and sucrose (the complete results are given in Table S1, available in IJSEM Online). Distinctively, *P. haikarae* DSM 16980^T and *P. cerevisiiphilus* DSM 20467^T were positive in β-galactosidase and β-glucosidase tests, whereas strain TY^T was negative (detailed API ZYM results are given in Table S2). Milk supported growth of TY^T, which is different from *P. frisingensis* DSM 6306^T (Juvonen & Suihko, 2006). Strain TY^T was sensitive to all tested antibiotics, while the other *Pectinatus* type strains could tolerate penicillin and, in most cases, vancomycin (see Table 1). H_2S production and the methyl red test were positive, while indole production, the Voges–Proskauer reaction and tests for catalase and oxidase activity were negative. After incubation for 24 h in medium YG, the main fermentation products of strain TY^T from glucose (5 g l^{-1}) were 0.2 g acetic acid and 1.3 g propionic acid l^{-1}, while strain *P. cerevisiiphilus* DSM 20467^T produced 0.2 g acetic acid and 1.0 g propionic acid l^{-1}.

Fatty acid methyl esters (FAMEs) were prepared from liquid culture when the OD_{600} reached 1.2, as described by Kuykendall *et al.* (1988). Identification and quantification were then performed by using the Sherlock Microbial Identification System (MIDI) with the standard MIS Library Generation software (anaerobe method; library BHIBLA 3.80). Fatty acid compositions of TY^T, *P. cerevisiiphilus* DSM 20467^T, *P. haikarae* DSM 16980^T and *P. frisingensis* DSM 6306^T are listed in Table 2. All four strains had similar fatty acid profiles except that the amounts of $C_{13:0}$, $C_{14:0}$, $C_{15:0}$, $C_{17:0}$ FAME, anteiso-$C_{18:1}$ and summed feature 5 ($C_{17:1}\omega7c$ and/or $C_{17:2}$) were different. The existence of quinones was checked by quinone extraction (Tindall, 1990) and LC-MS analysis [Agilent HC-C18; 75 % methanol and 25 % 2-propanol as the mobile phase at 0.8 ml min^{-1}, wavelength 270 nm, column temperature 40 °C (THERMO Finnigan LCQ DECA XP MAX)]. No methyl naphthoquinone or ubiquinone was detected in these strains. Polar lipids were extracted (Chung *et al.*, 2000) and analysed by two-dimensional TLC (Ferraz *et al.*, 1994) on silica gel 60 F_{254} plates (Merck). The result indicated that these strains had similar polar lipid components (Fig. S1). Polyamines were determined as described by Ducros *et al.* (2009). Putrescine and cadaverine were found in all strains.

Table 1. Characteristics of strain TY^T and type strains of other *Pectinatus* species

Strains: 1, TY^T; 2, *P. cerevisiiphilus* DSM 20467^T; 3, *P. haikarae* DSM 16980^T; 4, *P. frisingensis* DSM 6306^T. Strain TY^T also produced acid from galactose, glucose and fructose and was able to hydrolyse milk but failed to use gelatin. +, Positive; −, negative; W, weakly positive. All data were obtained in this study.

Characteristic	1	2	3	4
Isolation source	Pickle wastewater	Spoiled beer	Brewery bottling hall	Spoiled beer
Cell width × length (μm)	0.5–1.0 × 3–50	0.7–0.9 × 2–30	0.6–0.8 × 3–50	0.7–0.9 × 3–20
DNA G+C content (mol%)	35.9	38.6	39.1	38.4
Temperature for growth (°C)				
Range	10–40	10–45	15–30	15–37
Optimum	28	30	20–30	30
pH for growth				
Range	3.5–8.5	4.0–8.0	4.0–8.0	3.5–8.0
Optimum	6.5	6.5	7.0	6.5
NaCl concentration for growth (%)				
Range	0–3	0–1	0–1	0–2
Optimum	1	0	0	0
Acid production from:				
Glycerol	+	W	−	−
Aesculin	W	+	−	+
Lactose	−	−	+	−
D-Xylose	−	+	+	−
Mannose	−	+	+	+
Mannitol	+	−	+	+
Sucrose	+	−	−	−
Susceptibility to:				
Penicillin (10 IU)	+	−	−	−
Vancomycin (5 μg)	+	+	−	−
Enzyme activity				
β-Galactosidase	−	+	+	−
β-Glucosidase	−	+	+	+
Catalase activity	−	−	+	−
Acetoin production	−	+	+	+

Table 2. Whole-cell fatty acid components of strain TY^T and type strains of other *Pectinatus* species

Strains: 1, TY^T; 2, *P. cerevisiiphilus* DSM 20467^T; 3, *P. haikarae* DSM 16980^T; 4, *P. frisingensis* DSM 6306^T. Values are percentages of total FAMEs, and data were obtained in this study. tr, Trace (content <0.5 %); ND, not detected; DMA, dimethyl acetal; ECL, equivalent chain length. Values highlighted in bold differ by >5 % from the content in TY^T.

Fatty acid	1	2	3	4
Saturated				
$C_{11:0}$	13.3	10.4	10.1	13.3
$C_{12:0}$	tr	0.8	tr	0.6
iso-$C_{12:0}$	tr	tr	ND	ND
$C_{13:0}$	8.7	**3.6**	**15.7**	6.8
$C_{14:0}$ FAME	tr	1.7	0.8	0.7
$C_{14:0}$ DMA	18.7	**12.5**	16.3	16.3
$C_{15:0}$	15.4	**8.7**	**21.4**	13.0
$C_{16:0}$	tr	0.9	0.8	tr
$C_{17:0}$ FAME	1.8	1.1	**6.1**	2.6
$C_{17:0}$ DMA	1.3	0.7	3.0	1.6
Unsaturated				
$C_{14:1}\omega6c$	tr	1.9	0.6	tr
$C_{15:1}\omega5c/\omega8t$	tr	1.2	ND	ND
$C_{16:1}\omega8c$	4.0	5.7	2.1	3.7
$C_{16:1}\omega6c$	ND	0.9	ND	ND
$C_{17:1}\omega5c$	tr	0.7	1.6	1.3
anteiso-$C_{18:1}$	15.2	15.3	**7.4**	**8.4**
Unknown				
ECL 13.493	1.9	1.4	1.9	1.7
Summed features*				
1	ND	1.0	ND	0.4
2	6.2	7.7	4.4	3.3
3	0.6	0.6	0.7	1.0
4	ND	tr	ND	0.4
5	11.0	**21.2**	6.9	11.2

*Summed features are groups of two or three fatty acids that could not be separated by using the MIDI System. Summed feature 1 contained $C_{12:0}$ 3-OH and/or $C_{13:0}$; summed feature 2, contained one or more of unknown ECL 14.762, $C_{15:2}$ and $C_{15:1}\omega7c$; summed feature 3 contained $C_{15:0}$ and/or $C_{14:0}$ 3-OH; summed feature 4 contained anteiso-$C_{15:0}$ 3-OH and/or $C_{16:1}\omega8c$; summed feature 5 contained $C_{17:1}\omega7c$ and/or $C_{17:2}$.

Genomic DNA was extracted as described by Marmur (1961). The 16S rRNA gene (1470 bp) was amplified as described by Wu *et al.* (2010). Pairwise sequence alignment was performed with the EzTaxon service (Chun et *al.*, 2007). Multiple sequence alignment and maximum-likelihood analysis (Felsenstein, 1981), neighbour-joining analysis (Saitou & Nei, 1987) using Kimura's two-parameter model (Kimura, 1980) and maximum-parsimony analysis (Fitch, 1971) were conducted using MEGA 5.05 software (Tamura *et al.*, 2011). As shown in Fig. 2, though the 16S rRNA gene sequence similarities are relatively low (93.2–94.8 %), strain TY^T clustered in the genus *Pectinatus*. The DNA G+C contents as determined by HPLC (Mesbah & Whitman, 1989) were 35.9, 38.6, 39.1 and 38.4 mol% for TY^T, *P. cerevisiiphilus* DSM 20467^T, *P. haikarae* DSM 16980^T and *P. frisingensis* DSM 6306^T, respectively. DNA–DNA hybridization was performed with a DU800 spectrophotometer (Beckman Coulter) as described by De Ley *et al.* (1970). DNA–DNA relatedness between strain TY^T and *P. cerevisiiphilus* DSM 20467^T, *P. haikarae* DSM 16980^T and *P. frisingensis* DSM 6306^T was respectively 37.1, 46.4 and 31.2 %.

Based on the similar genotypic characteristics of DNA G+C content and the close phylogenetic relationship, as well as phenotypic characteristics such as the typical comb-like flagella and other similar physiological and chemical properties shown in Table 1, strain TY^T could be classified in the genus *Pectinatus*. On the other hand, several characteristics could be found that differentiated TY^T from other *Pectinatus* species with validly published names: for example, the low 16S rRNA gene similarity (<95 %), the low DNA–DNA relatedness (<50 %), sensitivities to penicillin and vancomycin, the inability to produce acetoin and the ability to produce acid from glycerol, sucrose, lactose, D-xylose, mannose and mannitol. Furthermore, to our knowledge, this is the first report of a *Pectinatus* strain from a salty environment. Therefore, according to the genotypic and phenotypic characteristics described above, strain TY^T is considered to represent a novel *Pectinatus* species, with the name *Pectinatus brassicae* sp. nov.

Description of *Pectinatus brassicae* sp. nov.

Pectinatus brassicae (bras.si′ca.e. L. gen. n. *brassicae* of cabbage, referring to the ingredients of pickle, from which the type strain was isolated).

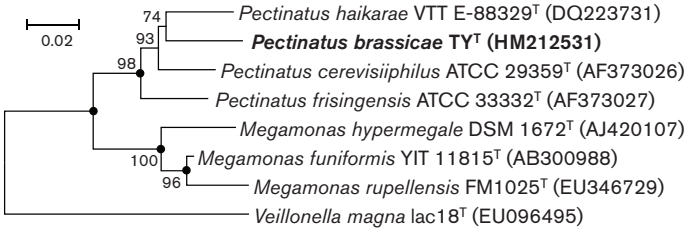

Fig. 2. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the systematic relationships of strain TY^T and related strains. Bootstrap values based on 1000 replications are shown; filled circles indicate nodes that were also supported by the maximum-likelihood method. Maximum-parsimony and maximum-likelihood trees are available in Fig. S2. Bar, substitutions per nucleotide position.

Cells are strictly anaerobic, Gram-negative, non-spore-forming, slightly curved, 0.5–1.0 × 3–50 μm. Plenty of inclusion granules that are not stained by Sudan black are produced in late-exponential and stationary phases. Growth occurs at 10–40 °C (optimum at 28 °C), at pH 3.8–8.5 (optimum at pH 6.5) and in 0–3 % NaCl (optimum at 1 %) in PYG medium. Colonies are papillary, maize-yellow and circular with entire margins, 0.5–2.0 mm in diameter after incubation on PYG plates for 2 days. Glucose fermentation produces mainly acetic and propionic acids. H_2S production and the methyl red test are positive, while indole production, the Voges–Proskauer test and nitrate reduction are negative. Acid phosphatase and naphthol-AS-BI-phosphohydrolase activities are strongly positive. The major fatty acids are $C_{14:0}$ DMA, $C_{15:0}$, anteiso-$C_{18:1}$, $C_{11:0}$ and summed feature 5 ($C_{17:1}\omega7c$ and/or $C_{17:2}$). Glycerol, sucrose, D-galactose, glucose, fructose and mannitol can be metabolized to acids.

The type strain is TY^T (=JCM 17499^T =DSM 24661^T). The DNA G+C content of the type strain is 35.9 mol%.

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