Pisciglobus halotolerans gen. nov., sp. nov., isolated from fish sauce

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Two strains of Gram-stain-positive, catalase-negative, tetrad-forming cocci, C01^T and C02, were isolated in Thailand from fish sauce. They were facultatively anaerobic, non-motile and non-spore-forming bacteria. These strains produced L-lactic acid from glucose. They grew at pH 5.0–9.0, at 15–40 °C and in the presence of 10 % (w/v) NaCl. The dominant fatty acid was C_{18:1ω9c}. The DNA G + C contents of strains C01^T and C02 were 38.6 and 38.7 mol%, respectively. Strain C01^T was related most closely to *Desemzia incerta* DSM 20581^T, with a 16S rRNA gene sequence similarity of 96.9 %. The strains could be distinguished clearly from *D. incerta* DSM 20581^T based on cell morphology, physiological and biochemical characteristics and low levels of DNA–DNA relatedness. On the basis of the data presented, strains C01^T and C02 are considered to represent a novel species of a new genus in the *Bacillus*–*Lactobacillus* cluster, for which the name *Pisciglobus halotolerans* gen. nov., sp. nov. is proposed. The type strain of *Pisciglobus halotolerans* is C01^T (=KCTC 13150^T =TISTR 1958^T =PCU 316^T).

Gram-positive, catalase-negative cocci in the genera *Aerococcus*, *Pediococcus*, *Lactococcus*, *Vagococcus*, *Enterococcus*, *Weissella*, *Oenococcus* and *Tetragenococcus* are widely distributed in plants, beverages, fermented foods, dairy products, faeces and water (Schleifer & Kilpper-Bälz, 1984; Schleifer *et al.*, 1985; Collins *et al.*, 1984, 1989, 1990, 1993; Dicks *et al.*, 1995). Most produce L-lactic acid from glucose, except for some species in the genera *Pediococcus*, *Weissella* and *Oenococcus* (Collins *et al.*, 1990; Dicks *et al.*, 1995; Holzapfel *et al.*, 2006). In addition, *Enterococcus* and *Weissella* strains are able to grow maximally in the presence of 10 % NaCl, whereas halophilic *Tetragenococcus* strains are able to grow with over 18 % NaCl. In the present study, we isolated Gram-stain-positive, catalase-negative and salt-tolerant tetrad-forming cocci from fermented fish sauce in Thailand. The strains grew maximally in the presence of

10 % (w/v) NaCl and produced L-lactic acid from glucose. On the basis of their phenotypic and chemotaxonomic characteristics, 16S rRNA gene sequence analysis and DNA–DNA hybridization data, they were differentiated from the closely related *Desemzia incerta* DSM 20581^T, and are considered to represent a novel species of a new genus in the *Bacillus*–*Lactobacillus* cluster.

Samples of fish mixed with salt to produce fermented fish sauce were collected from the early stage of fermentation at a factory in Chonburi Province, Thailand. Lactic acid bacteria were isolated by using a pouring-plate technique with MRS agar (De Man *et al.*, 1960) containing 5 % NaCl with incubation at 30 °C for 3–5 days. Cell morphology, cell size, cell arrangement and colonial appearance were examined on MRS agar incubated for 5 days. The Hucker–Conn modification was used for Gram staining (Hucker & Conn, 1923). Cell morphology and spore formation were observed by phase-contrast and scanning electron microscopy. Motility was detected by the appearance of stab cultures in soft agar (Whittenbury, 1963). Tests for catalase

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain C01^T is GU459067.

Two supplementary figures and a supplementary table are available with the online version of this paper.

with haematin in the medium, oxidase, nitrate reduction, reaction in litmus milk (Difco), hydrolysis of L-arginine, casein, starch, gelatin and tributyrin, oxidation–fermentation, hydrogen sulfide formation and methyl red and Voges–Proskauer reactions were performed as reported by Barrow & Feltham (1993) and Tanasupawat *et al.* (1992, 1998). Gas production from glucose was examined in MRS broth and a Durham tube was used for detecting gas. The effects of temperature (10, 40, 42 and 50 °C), starting pH (4.2, 5.0, 6.5, 8.0 and 9.0) and NaCl concentration (0, 10, 15, 20 and 25 %, w/v) were tested by using MRS broth as basal medium. Acid formation from carbohydrates was determined as reported previously (Tanasupawat *et al.*, 1998). All tests were carried out by incubating the cultures at 30 °C for 3–5 days, except for the investigation of effects of temperature. Additional biochemical characteristics were recorded after 2 days of incubation in API 50 CH strips. Lactic acid was extracted from the supernatant of the GYPB fermentation broth of strains (Tanasupawat *et al.*, 1998) by using diethyl ether and was analysed enzymically as reported by Okada *et al.* (1978). Analysis of total cellular fatty acids was performed with cells grown on trypticase soy agar (Difco) for 48 h at 30 °C, by using the standard Microbial Identification System (MIDI Inc.) for automated GC analysis (Sasser, 1990). TLC plates (Merck no. 5577) developed with the solvent system n-butanol/acetic acid/water (60:15:25, v/v) were used to determine lysine in the cell wall (Tanasupawat *et al.*, 1993).

DNA was isolated from cells grown in MRS broth after incubating for 1–2 days and was purified by the method of Saito & Miura (1963). DNA base composition was determined by reversed-phase HPLC (Tamaoka & Komagata, 1984). The 16S rRNA gene of strain C01^T was amplified and the PCR product was purified and sequenced as described previously (Tanasupawat *et al.*, 2004). The sequence of strain C01^T was aligned with selected sequences obtained from GenBank by using CLUSTAL X version 1.83 (Thompson *et al.*, 1997). The alignment was edited manually to remove gaps and ambiguous nucleotides prior to the construction of phylogenetic trees. Phylogenetic trees were constructed by using the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Kluge & Farris, 1969) and minimum-evolution (Felsenstein, 1997) methods in the MEGA program version 2.1. Confidence values of branches of the phylogenetic tree were determined by using bootstrap analyses (Felsenstein, 1985) based on 1000 resamplings. Levels of 16S rRNA gene sequence similarity were determined by using the EzTaxon server (Chun *et al.*, 2007). Nucleotide positions are given according to the *Escherichia coli* numbering system (Brosius *et al.*, 1978). DNA–DNA hybridization with photobiotin-labelled probes was carried out in 2 × SSC and 50 % formamide solution at 40 °C for 15 h (Ezaki *et al.*, 1989).

Cells of strains C0-1^T and C0-2 were Gram-positive, facultatively anaerobic, non-motile, non-spore-forming cocci, 0.6–1 µm in diameter, that were arranged in pairs,

tetrads or packets (Fig. 1). Colonies on MRS agar plates were 0.4–1.6 mm in diameter, circular, raised or of low convexity with entire margins and non-pigmented. Phenotypic characteristics are detailed in the genus and species descriptions and in Table 1. The strains were able to grow in the presence of 10 % (w/v) NaCl. Cell form and colonial appearance were similar to lactic acid bacteria in the genera *Pediococcus*, *Tetragenococcus* and *Aerococcus* (Collins *et al.*, 1990; Holzapfel *et al.*, 2006). Strains C0-1^T and C0-2 contained C_{18:1}ω9c (39.47–43.38 %) as the predominant cellular fatty acid. Their fatty acid profiles corresponded in general composition to that of *D. incerta* DSM 20581^T, but there were significant differences in the amounts of C_{16:0}, C_{16:1}ω9c and C_{18:1}ω7c, as reported by Stackebrandt *et al.* (1999) and this study (Table 2). The two novel strains also contained C_{10:0} (0.42–0.45 %), C_{12:0} (2.93–3.4 %), C_{14:0} (21.73–23.29 %), C_{16:1}ω9c (9.94–12.03 %), C_{16:0} (16.35–17.78 %), C_{18:1}ω7c (1.2–1.31 %), C_{18:0} (1.37–1.9 %) and C_{20:1}ω9c (trace) (Table 2). They contained L-Lys in the cell-wall peptidoglycan. The DNA G+C contents of strains C01^T and C02 were 38.6 and 38.7 mol%, respectively.

The almost-complete 16S rRNA gene sequence of strain C01^T (1501 nt) was compared against those of the type strains of all recognized species of the genus *Carnobacterium*, *D. incerta* and other selected representatives of the family *Carnobacteriaceae*. Phylogenetic analysis based on this large dataset revealed that the novel strains belonged to a cluster within the family *Carnobacteriaceae* (see Supplementary Figs S1 and S2, available in IJSEM Online). When the sequence of strain C01^T was compared with selected 16S rRNA gene sequences of members in the family *Carnobacteriaceae*, it formed a clade with *D. incerta* DSM 20581^T (Fig. 2). Strain C01^T shared highest levels of 16S rRNA gene sequence similarity with *D. incerta* DSM 20581^T (96.9 %), *Carnobacterium viridans* MPL-11^T (95.3 %), *Marinilactibacillus psychrotolerans* M13-2^T (91.8 %), *Isobaculum melis* CCUG 37660^T (93.9 %), *Granulicatella elegans* ATCC 700633^T (91.8 %), *Atopobacter phocae* m1590/94/2^T (92.4 %),

Fig. 1. Scanning electron micrograph of cells of strain C01^T grown on MRS agar for 3 days. Bar, 1 µm.

Table 1. Differential characteristics between strains C01^T and C02 and *D. incerta* DSM 20581^T

Strain C02 gave identical results to strain C01^T unless indicated. Data are from the present study except where indicated. w, Weakly positive.

Characteristic	Strain C01 ^T	<i>D. incerta</i> DSM 20581 ^T
Cell form	Cocci	Rods
Flagellation	None	One or two*
Growth in 10 % (w/v) NaCl	+	—
Acid production from:		
D-Galactose	+	—
Lactose	+	—
Mannitol	+	—
Methyl α -D-glucopyranoside	—	w
Starch	—	+
Turanose	w	—
2-Ketogluconate	—	w
5-Ketogluconate	—	+
DNA G + C content (mol%)	38.6/38.7†	40*

*Data from Stackebrandt *et al.* (1999).

†Values for strains C01^T/C02.

Trichococcus palustris DSM 9172^T (93.5 %) and *Vagococcus lutrae* m1134/97/1^T (94.3 %). The sequence of strain C01^T differed from those of its closest neighbours, the genera *Desemzia* (Stackebrandt *et al.*, 1999) and *Carnobacterium* (Collins *et al.*, 1987), in several signature positions (Supplementary Table S1), clearly differentiating the novel strains from both of these genera. In addition, strains C01^T and C02 showed 99.8 % DNA–DNA relatedness to each other but ≤ 5 % to *D. incerta* DSM 20581^T.

The two novel strains could be distinguished clearly from *D. incerta* DSM 20581^T based on cell form, physiological

and biochemical characteristics, cellular fatty acid compositions and DNA–DNA relatedness and 16S rRNA gene sequence data. In addition, they could be differentiated from the genera *Carnobacterium*, *Marinilactibacillus*, *Isobaculum*, *Granulicatella*, *Atopobacter*, *Trichococcus* and *Vagococcus* based on 16S rRNA gene sequence analyses as detailed above and as reported by Holley *et al.* (2002), Ishikawa *et al.* (2003), Lawson *et al.* (2000), Liu *et al.* (2002), Collins & Lawson (2000) and Collins *et al.* (1987, 2002). Therefore, strains C01^T and C02 are considered to represent a novel species of a new genus of the *Bacillus*–*Lactobacillus* cluster, for which the name *Pisciglobus halotolerans* gen. nov., sp. nov. is proposed.

Description of *Pisciglobus* gen. nov.

Pisciglobus [Pis.ci.glo'bus. L. n. *piscis* fish; L. masc. n. *globus* ball, sphere, globe; N.L. masc. n. *Pisciglobus* a sphere (coccus) from fish].

Cells are Gram-stain-positive, facultatively anaerobic, non-motile, non-spore-forming cocci, arranged in pairs, tetrads or packets. Colonies on MRS agar plates are circular, raised or of low convexity with entire margins and non-pigmented. Negative for catalase, gelatin hydrolysis and nitrate reduction. Ferment glucose but no gas is produced. Produce L-lactic acid from glucose. Tolerate up to 10 % (w/v) NaCl. C_{18:1 ω 9c} is the predominant fatty acid. The cell-wall peptidoglycan is of the L-Lys type. DNA G + C content of known strains is 38.6–38.7 mol%. Known strains have been isolated from fermented fish sauce. The type species is *Pisciglobus halotolerans*.

Description of *Pisciglobus halotolerans* sp. nov.

Pisciglobus halotolerans (ha.lo.to'le.rans. Gr. n. *hals* salt; L. pres. part. *tolerans* tolerating; N.L. part. adj. *halotolerans* salt-tolerating).

Has the following characteristics in addition to those given for the genus. Cells are 0.6–1 μ m in diameter. Positive for methyl red reaction and hydrolysis of L-arginine. Negative for oxidase, hydrolysis of casein, starch and tributyrin, Voges–Proskauer reaction and hydrogen sulfide formation. Produces catalase in medium containing haematin. No acid, coagulation, reduction or liquefaction is observed in litmus milk. Grows at pH 5.0–9.0, at 15–40 °C and in the presence of 10 % (w/v) NaCl. Acid is produced from D-amylgdalin, aesculin, arbutin, D-galactose, gentiobiose (weakly), D-glucose, N-acetylglucosamine, D-fructose, cellobiose, lactose, maltose, D-mannose, methyl α -D-glucoside, methyl α -D-glucopyranoside (weakly), D-mannitol, D-ribose, sucrose, salicin and trehalose, but not from adonitol, D- or L-arabinose, dulcitol, erythritol, gluconate, glycogen, glycerol, methyl α -D-mannopyranoside, methyl β -xyloside, inositol, inulin, melibiose, melezitose, D- or L-xylose, rhamnose, D-sorbose, starch, xylitol, turanose, D-lyxose, D-tagatose, D- or L-fucose, D- or L-arabitol,

Table 2. Cellular fatty acid profiles of strains C01^T and C02 and *D. incerta* DSM 20581^T

Data were obtained in this study. Values are percentages of total fatty acids. ND, Not detected.

Fatty acid	C01 ^T	C02	<i>D. incerta</i> DSM 20581 ^T
C _{10:0}	0.42	0.45	0.87
C _{12:0}	2.93	3.4	3.54
C _{14:0}	23.29	21.73	21.67
C _{16:1ω9c}	12.03	9.94	28.21
C _{16:0}	17.78	16.35	28.74
C _{18:1ω9c}	39.47	43.38	7.58
C _{18:1ω7c}	1.31	1.2	0.41
C _{18:0}	1.37	1.9	1.37
C _{20:1ω9c}	0.38	ND	4.25

Fig. 2. Neighbour-joining tree (Saitou & Nei, 1987) based on almost-complete 16S rRNA gene sequences showing the relationships between strain C01^T and the type strains of related members of the family *Carnobacteriaceae*. Numbers at nodes are bootstrap values (percentages of 1000 resamplings). Bar, 0.01 substitutions per nucleotide position.

2-ketogluconate, 5-ketogluconate, D-sorbitol or raffinose. The DNA G + C content of the type strain is 38.6 mol%.

The type strain, C01^T (=KCTC 13150^T =TISTR 1958^T =PCU 316^T), was isolated from fermented fish sauce produced in Thailand. Strain C02, isolated from a similar source, is a second strain of the species.

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