Pichia thermomethanolica sp. nov., a novel thermotolerant, methylotrophic yeast isolated in Thailand

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Three strains (N002, N069 and PT31^T) of a novel thermotolerant methylotrophic yeast species belonging to the genus *Pichia* were isolated from soil collected in Thailand by three consecutive enrichments in methanol broth at room temperature. They were categorized as thermotolerant strains on the basis of their good growth below 20 °C and up to a high temperature (37 °C). The major characteristics of the three strains included the following and placed them in the genus *Pichia*: the formation of four helmet-/hat-shaped ascospores in a deliquescent ascus that might be unconjugated or produced by conjugation between a cell and its bud or between independent cells; multilateral budding; the presence of ubiquinone Q-7; negative for Diazonium blue B colour and urease reactions; and the absence of arthrospores and ballistospores. The three strains differed by one to three nucleotide substitutions in the sequences of the D1/D2 domain of the large-subunit rDNA sequence. Phylogenetic analysis revealed that their closest species was *Pichia dorogensis*, but with 11–13 nucleotide substitutions in 554 nt. The phenotypic characteristics of the three strains were the same. The strains could be distinguished from *P. dorogensis* by a number of phenotypic characteristics. On the basis of the above findings, these three strains were assigned to a single novel species of *Pichia*, for which the name *Pichia thermomethanolica* sp. nov. is proposed. The type strain is PT31^T (= BCC 16875^T = JCM 12984^T = CBS 10098^T).

Methanol metabolism is a characteristic shown by relatively few yeast species (the methylotrophic yeasts); most known methylotrophic yeasts belong to the genera *Pichia* and *Candida* (Kurtzman & Fell, 1998; Barnett *et al.*, 2000). From the differences in the partial sequences of small-subunit (18S) and large-subunit (26S) rDNA sequence, Yamada *et al.* (1994) proposed the transfer of hat-shaped ascospore-forming, nitrate-assimilating methylotrophic yeast species formerly classified in the genus *Pichia* (*Pichia angusta*, *Pichia minuta* var. *minuta*, *P. minuta* var. *nonfermentans*, *Pichia philodendra*, *Pichia glucozyma* and *Pichia henricii*) to a newly described genus, *Ogataea*. Thereafter, Mikata & Yamada (1995) transferred *Pichia kodamae* to the genus *Ogataea*.

Recently, Morais *et al.* (2004) described *Ogataea falcaomraisi* as a novel sporogenous methylotrophic yeast, on the basis of the sequences of the D1/D2 domain of the 26S rDNA. However, the circumscription of *Ogataea* is not generally accepted. Kurtzman & Robnett (1998) suggested that a more robust dataset is required in order to substantiate the circumscription.

Methylotrophic yeasts have attracted interest since they were first isolated for both physiological study and industrial applications (Levine & Cooney, 1973; Demain *et al.*, 1998). Recently, some of them have become the principal biocatalyst for the production of useful compounds, and are an important host for the expression of genes (Sakai *et al.*, 1996; Gellissen, 2000). Thermotolerant or thermophilic micro-organisms have certain advantages over mesophiles in industrial processes. Thermophilic yeasts are defined as yeasts with a minimal temperature for growth of 20 °C, but with no maximal temperature limit for growth (Arthur &

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Watson, 1976; Watson, 1987). By this definition, a yeast strain that grows below 20 °C, e.g. 10 °C, and up to high temperatures, such as 37–48 °C, is considered to be thermotolerant (Arthur & Watson, 1976).

In the course of an investigation of thermotolerant methylotrophic yeasts in Thailand, 253 strains were isolated from 634 samples of soil and plant materials (e.g. flowers, fruits, bark and tree exudates) by a technique involving three consecutive methanol enrichments (Limtong *et al.*, 2004). Fifty-four strains that showed good growth at 10 °C as well as at 37 °C were categorized as thermotolerant strains. Of these, four strains have previously been reported as representing three novel species, *Pichia siamensis*, *Candida krabiensis* and *Candida sithepensis* (Limtong *et al.*, 2004). In this study, we describe another three of these strains (N002, N069 and PT31^T) obtained from soil samples as a novel thermotolerant, methylotrophic species of the genus *Pichia*.

Strain N002 was isolated from soil collected in Saraburi Province, while N069 and PT31^T were obtained from two soil samples in Pathalung Province. The isolation was carried out using a procedure involving three consecutive enrichments, with 1 % methanol-YNB broth (0.67 % Difco yeast nitrogen base and 1 %, v/v, methanol), at room temperature as previously described (Limtong *et al.*, 2004). The strains were categorized as thermotolerant methylotrophic yeasts on the basis of their good growth at 10 and 37 °C.

The strains were characterized morphologically, physiologically and biochemically by using the standard methods described by Yarrow (1998). Assimilation of nitrogen compounds was examined on solid media with starved inocula, according to the method of Nakase & Suzuki (1986). Growth at various temperatures was determined by cultivation on YM broth (3 g yeast extract l⁻¹, 3 g malt extract l⁻¹, 5 g peptone l⁻¹, 10 g glucose l⁻¹) and YM agar (YM broth containing 20 g agar l⁻¹), using a water bath and an incubator, respectively. Ubiquinones were extracted from intact packed cells and purified according to the method described by Nakase & Suzuki (1986). The isoprenologues were identified by an HPLC system (Agilent 1100) using a Cosmosil (Waters 5C18) 4.6 × 250 mm column and methanol/isopropanol (2:1) at 1 ml min⁻¹ as the elution system, with spectrophotometric detection (wavelength 275 nm).

The sequences of the D1/D2 domains of the 26S rDNA of the three strains were determined by the National Collection of Industrial, Marine and Food Bacteria (Japan), as described previously (Limtong *et al.*, 2004). The sequences were compared pairwise by using a BLAST homology search (DNA Data Bank of Japan, Research Organization of Information and Systems, National Institute of Genetics) and were aligned with the sequences of related species using the multiple-alignment program CLUSTAL W, version 1.81 (Thompson *et al.*, 1997). A phylogenetic tree was constructed from the evolutionary distance data according

to the two-parameter method of Kimura (1980) and the neighbour-joining method (Saitou & Nei, 1987). Confidence limits for the phylogenetic tree were estimated from bootstrap analysis (1000 replicates) (Felsenstein, 1985). The sequences of the D1/D2 domains of the 26S rDNA revealed that the degree of similarity among the three strains ranged from 99.8 % (one substitution in 566 nt) to 99.5 % (three substitutions in 566 nt). This degree of sequence similarity implied that the three strains were conspecific.

In the phylogenetic tree based on the D1/D2 domains of 26S rDNA sequences, the three strains were in the same group and clustered with *Pichia dorogensis*, *P. kodamae*, *P. minuta* var. *nonfermentans* and *P. minuta* var. *minuta* (other methylotrophic yeasts) (Fig. 1). The closest species, in terms of pairwise sequence similarity to the three strains N002, N069 and PT31^T, was *P. dorogensis* but with differences of 11, 13 and 13 nucleotide substitutions, respectively, in 554 nt of the D1/D2 domain of the 26S rDNA. According to Kurtzman & Robnett (1998), yeasts strains that show nucleotide substitution greater than 1 % in the D1/D2 domain of the 26S rDNA are likely to represent different species.

The three strains formed four helmet-/hat-shaped ascospores in a deliquescent ascus that might be unconjugated or produced by conjugation between a cell and its bud or between independent cells (Fig. 2), proliferated by multi-lateral budding, lacked arthrospores and ballistospores, were negative for Diazonium blue B colour and urease reactions and had Q-7 as the major ubiquinone. These characteristics coincided well with those of the genus *Pichia*. The strains also shared the same taxonomic characteristics, as shown in Table 1. We therefore conclude that the three strains represent a single novel species of *Pichia*. The name *Pichia thermomethanolica* sp. nov. is proposed for these strains.

In practice, *P. thermomethanolica* can be distinguished from *P. dorogensis*, the closest species in the phylogenetic tree, by using a number of phenotypic characteristics, as shown in Table 1.

Latin diagnosis of *Pichia thermomethanolica* Limtong, Srisuk, Yongmanitchai, Yurimoto, Nakase et Kato sp. nov.

In agaro YM post dies 3–5 ad 25 °C cellulae spheroidae aut ovoideae (0.8–4.2 × 1.7–5.0 µm), singulae, aut binae, per germinationem multipolarem reproducentes. Cultura albida, glabra, nitida, butyrosa, margine glabra. Pellicula non formatur. Mycelium nec pseudomycelium non formantur. Ascosporae galeiformes aut pileiformes, 4 in ascum. Glucosum fermentatur at non galactosum, maltosum, sucrosus, lactosum nec raffinosis. Glucosum, L-sorbosum, D-xylosum, D-ribosum, D-arabiosum, L-rhamnosum, sucrosus, maltosum, trehalosum, α-methyl-D-glucosidum, cellobiosum, salicinum, melezitosisum, glycerolum, erythritolum, ribitololum, D-glucitololum, D-mannitololum, glucono-δ-lactonum, acidum

Fig. 1. Phylogenetic tree, based on D1/D2 domains of the 26S rDNA, showing the positions of the three novel strains with respect to closely related species. The phylogenetic tree was constructed by using the neighbour-joining method, based on approximately 600 nt, according to the Kimura two-parameter system. Numbers indicate percentages of bootstrap sampling, derived from 1000 samples.

D-gluconicum, acidum succinicum, acidum citricum, methanolum et ethanolum assimilantur at non galactosum, N-acetyl-D-gluosaminum, L-arabinosum, melibiosum, lactosum, raffinsum, inulinum, amylum solubile, galactitolum, acidum 2-ketogluconicum, acidum 5-ketogluconicum, acidum DL-lacticum, acidum D-glucuronicum, acidum D-galacturonicum

nec inositolum. Nitricum, nitrosum, ethylaminum, L-lysinum et cadaverinum assimilantur. Crescere potest in temperatura 40 °C at non in 45 °C. Crescit in 0.1 % cycloheximido, 50 % glucosum et 10 % NaCl/5 % glucosum. Non crescit in 60 % glucosum. Ureum non hydrolysatur. Diazonium caeruleum B non respondens. Ubiquinonum majus: Q-7.

Fig. 2. *P. thermomethanolica* sp. nov. grown on YM agar for 3 days at 25 °C. (a) Vegetative cells and ascospores of strain PT31^T; (b) ascospores of strain PT31^T; (c) vegetative cells of strain N002; (d) vegetative cells and ascospores of strain N002. Bar, 10 μ m.

Table 1. Phenotypic characteristics of *P. dorogensis* and *P. thermomethanolica* sp. nov. strains

Strains N002 and N069 gave identical results to strain PT31^T. All strains were negative for the fermentation of D-galactose, maltose, sucrose, lactose and raffinose. D-Glucose, L-sorbose, D-xylose, D-ribose, D-arabinose, α-trehalose, cellobiose, salicin, glycerol, erythritol, ribitol, D-glucitol, D-mannitol, D-glucono-1,5-lactone, succinic acid, citric acid, methanol, ethanol, ethylamine hydrochloride, L-lysine hydrochloride and cadaverine were assimilated by all strains, but D-galactose, N-acetyl-D-glucosamine, L-arabinose, melibiose, lactose, raffinose, inulin, soluble starch, galactitol, myo-inositol, 2-ketogluconic acid, 5-ketogluconic acid, DL-lactic acid, D-glucuronic acid and D-galacturonic acid were not. In all cases, growth in vitamin-free medium was negative, growth at 37 °C was positive, and growth at 45 °C was negative. Growth on medium containing 0.1% cycloheximide was positive in all cases. Diazonium blue B colour and urease reactions were negative in all cases. Symbols: +, positive; −, negative; D, delayed positive; ND, no data available; S, slow; W, weak.

Characteristic	<i>P. thermo- methanolica</i>	<i>P. dorigensis</i>
Fermentation of glucose	+	S
Assimilation of:		
L-Arabinose	−	D
L-Rhamnose	+	−
Sucrose	+	−
Maltose	+	−
Methyl α-D-glucoside	+	−
Melezitose	+	−
D-Gluconic acid	+	−
Potassium nitrate	+	−
Sodium nitrite	+	−
Growth		
At 40 °C	+	W
With 50 % glucose	+	−
With 60 % glucose	−	ND
With 10 % NaCl + 5 % glucose	+	−
Liquefaction of gelatin	+	ND

Typus stirpis PT31^T (=BCC 16875^T=JCM 12984^T=CBS 10098^T) *isolatus ex solo, in Pathalung Provincia, Thailandia, conservatur in collectionibus culturarum quas* BIOTEC Culture Collection, BIOTEC Central Research Unit (Pathumthani, Thailand), Japan Collection of Micro-organisms, RIKEN (Saitama, Japan) *et* Centraalbureau voor Schimmelcultures (Utrecht, The Netherlands) *deposita est.*

Description of *Pichia thermomethanolica* Limtong, Srisuk, Yongmanitchai, Yurimoto, Nakase & Kato sp. nov.

Pichia thermomethanolica (ther.mo.me.tha.no'li.ca. Gr. fem. n. *therme* heat; N.L. fem. adj. *methanolica* of methanol or methanol-assimilating; N.L. fem. adj. *thermomethanolica* thermotolerant and methanol-assimilating).

After growth on YM agar for 3–5 days at 25 °C, cells are spheroidal/spherical to ovoid (0.8–4.2 × 1.7–5.0 μm) and occur singly or in pairs (Fig. 2). Budding is multilateral. Streak culture is white to slightly tannish-white, smooth, glistening, butyrous and has an entire margin. A pellicle is not present during growth on the surface of assimilation media. Pseudohyphae and true hyphae are not produced in Dalmau plate culture on cornmeal agar after 7 days at 25 °C. Neither arthrospores nor ballistospores are produced. Forms four helmet-/hat-shaped ascospores in a deliquescent ascus that might be unconjugated or produced by conjugation between a cell and its bud or between independent cells (Fig. 2). Ascospores are observed on 5 % malt extract agar and YM agar after 3–7 days at 25 °C. The major ubiquinone is Q-7. Phenotypic characteristics of the species are shown in Table 1.

The type strain, PT31^T (=BCC 16875^T=JCM 12984^T=CBS 10098^T), was isolated from soil collected in Pathalung Province, Thailand.

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