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

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Three strains (N002, N069 and PT31T) 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 PT31T (=BCC 16875T = JCM 12984T = CBS 10098T).

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 falcamosraisii 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 &
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 krabienensis and Candida sithepensis (Limtong et al., 2004). In this study, we describe another three of these strains (N002, N069 and PT31T) 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 PT31T 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) inocula, according to the method of Nakase & Suzuki (1986). Growth at various temperatures was determined inocula, according to the method of Nakase & Suzuki (1986). Assimilation of nitrogen logarithmically and biochemically by using the standard methods described by Yarrow (1998). The strains were characterized morphologically, physiologically and biochemically by using the standard methods described by Yarrow (1998). 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 SC18) 4.6 x 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 PT31T, 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 standard 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**

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In agar YM post dies 3–5 ad 25 °C cellulae spheroidae aut ovoidae (0-8–4-2 x 1-7–5-0 μm), singulae, aut binae, per germinationem multipolare tandem reproducentes. Cultura albida, glabra, nitida, butyrosa, marginae glabra. Pellicula non formatur. Mycelium nec pseudomycelium nec formantur. Ascosporae galeiformes aut pileiformes, 4 in ascum. Glucosum fermentatur at non galactosum, maltosum, sucrosum, lactosum nec raffinicos. Glucosum, L-sorbosum, D-xylosum, D-ribosum, D-arabinosum, L-rhamnosum, sucrosum, maltosum, trehalosum, α-methyl-D-glucosidum, cellobiosum, salicinum, melezitosum, glycerolum, erythritolium, ribitolium, D-glucitolium, D-mannitolum, 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.

**Fig. 2.** *P. thermomethanolica* sp. nov. grown on YM agar for 3 days at 25 °C. (a) Vegetative cells and ascospores of strain PT31T; (b) ascospores of strain PT31T; (c) vegetative cells of strain N002; (d) vegetative cells and ascospores of strain N002. Bar, 10 μm.
Table 1. Phenotypic characteristics of *P. dorogensis* and *P. thermomethanolica* sp. nov. strains

<table>
<thead>
<tr>
<th>Characteristic</th>
<th><em>P. thermomethanolica</em></th>
<th><em>P. dorogensis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Fermentation of glucose</td>
<td>+</td>
<td>S</td>
</tr>
<tr>
<td>Assimilation of:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Arabinose</td>
<td>−</td>
<td>D</td>
</tr>
<tr>
<td>L-Rhamnose</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sucrose</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Maltose</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Methyl α-D-glucoside</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Melzitose</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>D-Gluconic acid</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Potassium nitrate</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Sodium nitrite</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Growth</td>
<td></td>
<td></td>
</tr>
<tr>
<td>At 40 °C</td>
<td>+</td>
<td>W</td>
</tr>
<tr>
<td>With 50 % glucose</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>With 60 % glucose</td>
<td>−</td>
<td>ND</td>
</tr>
<tr>
<td>With 10 % NaCl + 5 % glucose</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Liquefaction of gelatin</td>
<td>+</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Typus stirpis* PT31<sup>+</sup> (≡BCC 16875<sup>T</sup> ≡ JCM 12984<sup>T</sup> ≡ CBS 10098<sup>T</sup>) isolatus ex solo, in Pathalung Provincia, Thailandia, conservatur in collectionibus culturarum quas BIOTEC Culture Collection, BIOTEC Central Research Unit (Pathumthani, Thailand), Japan Collection of Microorganisms, 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.thermo.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 x 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<sup>+</sup> (≡BCC 16875<sup>T</sup> ≡ JCM 12984<sup>T</sup> ≡ CBS 10098<sup>T</sup>), was isolated from soil collected in Pathalung Province, Thailand.

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**References**


