Candida mengyuniaie sp. nov., a metsulfuron-methyl-resistant yeast

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A metsulfuron-methyl-resistant yeast strain, JHL^T, was isolated from metsulfuron-methyl-contaminated soil collected in Jiangsu Province, China. Through morphological and physiological analysis as well as a molecular phylogenetic analysis based on the 26S rRNA gene D1/D2 region and internal transcribed spacer (ITS), this strain, which forms a clade with *Candida vartiovaarae* and a teleomorphic species, *Williopsis saturnus*, was revealed to represent a novel species in the genus *Candida*. The name *Candida mengyuniaie* sp. nov. (type strain JHL^T=CGMCC 2.3681^T=CBS 10845^T) is proposed for this novel species.

Sulfonylurea herbicides, a class of acetolactate synthase (ALS)-inhibiting herbicides with extremely high biological activities, are widely used for weed control. Some of them, such as metsulfuron-methyl, chlorsulfuron and ethametsulfuron-methyl, persist for a long time in the environment. The residues of sulfonylurea herbicides in soils and water may have potential impacts on the environment and may damage sensitive crops planted in crop rotations. Sulfonylurea herbicides prevent the growth of plants, fungi or bacteria by inhibiting the ALS enzyme, which catalyses the common initial step in the biosynthesis of the branched amino acids isoleucine, leucine and valine (Blair & Martin, 1988; Brown, 1990). One of the main strategies to avoid herbicide damage is to plant transgenic herbicide-resistant crops (Ott *et al.*, 1996). Microbes constitute a potential and valuable source for sourcing sulfonylurea herbicide-resistant ALS genes (Pang *et al.*, 2003; Hill & Duggleby, 1998).

In this study, we isolated sulfonylurea herbicide-resistant yeast and bacterial strains from metsulfuron-methyl-contaminated soil samples by an enrichment method using minimal medium M9 containing 5 g glucose l⁻¹ as the carbon source and 1000 mg metsulfuron-methyl l⁻¹ as the selective pressure. Soil samples were collected from fields, which were often soaked with discharges from metsulfuron-methyl manufacturing facilities. Several metsulfuron-methyl-resistant micro-organisms were isolated and identified, including 60 bacteria and eight yeasts. The bacteria strains were identified as members of the genera *Pseudomonas*, *Klebsiella*, *Enterobacter* and *Salmonella* and

the yeast strains were initially identified as members of the genera *Pichia*, *Rhodotorula*, *Candida* and *Cryptococcus*. One yeast strain, JHL^T, showed differences from recognized yeast species in the 26S rRNA gene D1/D2 domain, ITS region and in morphological and physiological characteristics.

We tested the resistance of strain JHL^T, *Saccharomyces cerevisiae* and many other strains of closely related genera to four types of ALS-inhibiting herbicides, including metsulfuron-methyl, penoxsulam, imazethapyr and pyribenzoxim (Table 1). The results showed that, on the whole, strain JHL^T was more resistant to ALS-inhibiting herbicides than the other yeast strains tested.

Strain JHL^T was cultivated at 28 °C in YPD medium or M9 medium containing 5 g glucose l⁻¹ as a carbon source and 1000 mg metsulfuron-methyl l⁻¹ as selective pressure and was subsequently stored in 15 % glycerol at -70 °C. Morphological, physiological and biochemical characteristics of the novel strain were examined according to standard methods (Wickerham, 1951; Yarrow, 1998).

Total DNA was extracted from cells of strain JHL^T according to the method of Makimura *et al.* (1994). The 18S rRNA gene was amplified with the primer pair NS0 (5'-TATCTGGTTGATCCTGCC-3') and ITS2 (5'-GCTGCGTTCTTCATCGATGC-3') (Sterflinger & Prillinger, 2001). Purified PCR product was ligated into the linear vector pMD18-T (TaKaRa Biotechnology) and was sequenced with primer RV-M, primer 13-47, designed according to the sequence of vector pMD18-T, and another primer (5'-CGAGGAACAATTGGAGGGC-3') designed according to the 18S rRNA gene. A DNA fragment covering the ITS, 5.8S rRNA gene and 26S rRNA gene D1/D2 domain was amplified with the primer pairs ITS1 and NL4, the ITS region was sequenced directly with ITS1 and ITS4, the D1/D2 domain was sequenced directly with NL1 and NL4 (Lu *et al.*, 2004). These sequences were

Abbreviations: ALS, acetolactate synthase; CBS, Centraalbureau voor Schimmelfcultures, Fungal and Yeast Collection, Utrecht, The Netherlands; CICC, China Center of Industrial Culture Collection; ITS, internal transcribed spacer.

The GenBank/EMBL/DDBJ accession numbers for the 18S rRNA gene, 26S rRNA gene D1/D2 domain and ITS region sequences of JHL^T are EU043157, EU043158 and EU043159, respectively.

Table 1. Resistance (IC₅₀) of *Candida mengyuni* sp. nov. JHL^T and strains of closely related genera to different ALS-inhibiting herbicides

IC₅₀, the half maximal inhibitory concentration (mg l⁻¹), represents the concentration that is required for 50 % growth inhibition of the tested yeast strains by various herbicides. Strains: 1, *Candida mengyuni* sp. nov. JHL^T; 2, *Saccharomyces cerevisiae* CICC 1001; 3, *Williopsis saturnus* CICC 31231; 4, *Candida shehatae* CBS 5813^T; 5, *Pichia farinosa* CICC 1688. Growth was monitored by OD₆₂₀.

Herbicide	1	2	3	4	5
Metsulfuron-methyl	>5000	5	200	10	200
Penoxsulam	>5000	10	200	10	50
Imazethapyr	>5000	200	5000	1000	>5000
Pyribenzoxim	200	100	200	200	100

compared with known sequences in the GenBank database using BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/>). The D1/D2 domain sequence was aligned with closely related sequences using the CLUSTAL_X program (Thompson *et al.*, 1997). A phylogenetic tree was constructed from evolutionary distance data that were calculated with Kimura's two-parameter model (Kimura, 1980) by using the neighbour-joining method (Saitou & Nei, 1987). Bootstrap analysis (Felsenstein, 1985) was performed on 1000 random resamplings. Reference sequences were retrieved from GenBank with the accession numbers as indicated in the tree.

Sequence analysis

Previous studies have shown that strains with greater than 1 % substitutions in the D1/D2 domain usually represent separate species (Kurtzman & Robnett, 1997, 1998; Fell *et al.*, 2000). Sequence analysis of the ITS region is also a useful tool for yeast species identification, with a similar amount of intraspecific variation (James *et al.*, 1996; Sugita *et al.*, 1999; Bai *et al.*, 2001, 2002; Scorzetti *et al.*, 2002; Kurtzman & Robnett, 2003). Combined sequence analysis of the D1/D2 and ITS regions has been recommended for species identification (Scorzetti *et al.*, 2002).

The D1/D2 sequences from type strains of closely related taxa were selected for phylogenetic analysis based on the result of a BLAST search through GenBank with the D1/D2 sequence of strain JHL^T as the query (Fig. 1). The JHL^T strain formed a clade with *Candida vartiovaarae* and the five varieties of *Williopsis saturnus* (*Williopsis saturnus* var. *saturnus*, var. *mrakii*, var. *sargentensis*, var. *suaveolens* and var. *subsufficiens*). The D1/D2 sequence of strain JHL^T differed from that of the five varieties of *W. saturnus* by 3.3–3.8 % (14–17 substitutions, 5 gaps) and from that of *C. vartiovaarae* by 4.7 % (19 substitutions, 8 gaps). The closest type strains were *W. saturnus* var. *saturnus*, var. *sargentensis* and var. *suaveolens*, which differed from strain JHL^T by 3.3 % (14 substitutions, 5 gaps). Therefore, in the light of the number of nucleotide substitutions in the D1/D2 domain, strain JHL^T is considered to be distinct from any previously published yeast species.

Fig. 1. Neighbour-joining tree depicting the phylogenetic relationship of *Candida mengyuni* sp. nov. JHL^T with related species based on the 26S rRNA gene D1/D2 domain sequences. Bootstrap values >50 % from 1000 bootstrap replicates are shown at the branch points. Bar, 0.02 substitutions per site. Reference sequences were retrieved from GenBank under the accession numbers indicated.

The ITS region of strain JHL^T was sequenced and compared with that of the five *W. saturnus* varieties. In this region, the sequence of strain JHL^T differed from those of *W. saturnus* by more than 25 % and 30 % in the ITS1 and ITS2 regions, respectively. The ITS sequence of *C. vartiovaarae* is not yet available. The result of ITS comparative analysis indicated that strain JHL^T could be readily distinguished from currently recognized yeast species.

18S rRNA gene sequence analysis confirmed the close phylogenetic relationship of strain JHL^T with the *W. saturnus* complex and *C. vartiovaarae*.

Morphology and physiology

As strain JHL^T was most closely related to the teleomorphic species *W. saturnus*, special efforts were made to induce its sexual state. Most species of the genus *Williopsis* form asci and ascospores on 5 % malt extract agar at 25 °C after 1–3 weeks (Kurtzman, 1998a). However, a sexual state was not observed in cultures of strain JHL^T on this medium or on other media, including cornmeal agar and acetate agar.

Strain JHL^T differed remarkably from *W. saturnus* on the basis of its morphological and physiological characteristics. Colonies of strain JHL^T were smooth and convex on YPD agar, while colonies of *W. saturnus* were plicate and rough. Physiologically, strain JHL^T differed from *W. saturnus* in the assimilation reactions of D-galactose, L-rhamnose, sucrose, trehalose, methyl α -D-glucoside, cellobiose, salicin, arbutin, raffinose, inulin, xylitol, D-glucitol, succinate, nitrate, nitrite and L-lysine, by growth on medium without vitamins and by the higher maximum growth temperature (45 °C). Strain JHL^T differed from *C. vartiovaarae* in its inability to ferment maltose, its inability to assimilate maltose, cellobiose, salicin, arbutin, melezitose, xylitol and succinate, its ability to assimilate D-galactose, raffinose and inulin, and by the higher maximum growth temperature. A summary of physiological and other characteristics is given in Table 2.

Molecular, morphological and physiological comparisons demonstrated that strain JHL^T represents a novel species in the genus *Candida* Berkhout (Kurtzman, 1998b; Meyer *et al.*, 1998), for which we propose the name *Candida mengyuniiae* sp. nov.

Table 2. Physiological characteristics of strain JHL^T compared with closely related strains

Strains: 1, *C. mengyuniiae* sp. nov. JHL^T; 2, *W. saturnus* var. *saturnus* CBS 5761^T; 3, *W. saturnus* var. *mrakii* CBS 1707^T; 4, *W. saturnus* var. *sargentensis* CBS 6342^T; 5, *W. saturnus* var. *suaveolens* CBS 255^T; 6, *W. saturnus* var. *subsufficiens* CBS 5763^T; 7, *C. vartiovaarae* CBS 4289^T. Data for reference species were taken from the CBS website (<http://www.cbs.knaw.nl/yeast/BioloMICS.aspx>). +, Positive; –, negative; w, weakly positive; D, delayed positive.

Characteristic	1	2	3	4	5	6	7
Fermentation of:							
Maltose	–	–	–	–	–	–	+
Assimilation of:							
Arbutin	–	+	+	+	+	+	+
Cellobiose	–	+	+	+	+	+	+
D-Galactose	+	–	–	–	–	–	–
D-Glucitol	+	D	+	D	W, D, +*	D	+
Inulin	+	D	–	–	+	–	–
L-Lysine	+	+	+	–	+	+	+
Maltose	–	–	–	–	–	–	+
D-Mannitol	+	D	D	D	D	D	+
Melezitose	–	–	–	–	–	–	+
Methyl α -D-glucoside	+	–	–	–	–	–	+
Nitrate	+	+	+	–	+	+	+
Nitrite	+	+	+	–	+	+	+
Raffinose	+	+	–	–	+	+	–
L-Rhamnose	–	+	+	W, D, +*	–	+	–
Salicin	–	+	+	+	+	+	+
Succinate	–	+	+	+	+	+	+
Sucrose	+	+	–	–	+	+	+
Trehalose	+	–	–	–	–	–	+
Xylitol	–	+	–	D	+	D	+
Growth:							
Without vitamins	+	+	+	–	+	–	+
At 35 °C	+	–	NA	+	+	NA	–
At 45 °C	+	–	–	–	–	–	–

*Variable reaction with different testing methods.

Latin diagnosis of *Candida mengyuniae* He et Chen sp. nov.

In medio liquido YPD post dies 3 ad 28 °C, cellulae ellipsoideae vel elongatae (2.5–5.0 × 3.0–7.2 µm), cellulae singulae, binae et aggregatae. Per gemmationem multipolarem reproducentes. Post dies 3 sedimentum formatur. In agar YPD post dies 3 ad 28 °C, butyrosa, candida vel crenea, glabra, paucis hebia, convexa, margo glabro vel undulato. In agar farinae Zea mays post dies 7 ad 25 °C, pseudohyphae fiunt. Ascomata nulla. Glucosum, sucrosum, raffinsum (infirme), stachyosum, D-mannitolum et D-arabinum fermentatur, sed non D-galactosum, maltosum, trehalosum, melibiosum, cellobiosum, melezitium, inulinum et amylum. Glucosum, D-galactosum, D-xylosum, sucrosum, methyl α-D-glucosidum, melibiosum, raffinsum, inulinum, glycerolum, D-glucitolum, D-mannitolum et acidum citricum assimilantur, sed non D-glucosaminum, D-ribosum, D-arabiosum, D-arabiosum, L-rhamnosum, maltosum, cellobiosum, salicinum, arbutinum, melezitium, amylum, erythritolum, ribitolum, xylitolum, inositolum et gentiobium. Trehalosum assimilantur. Ammonium sulfatum, kalium nitricum, natrum nitrosum et L-lysinum assimilantur. Sine vitaminis externis supplementis crescens. Non crescit in 50 % glucose. Crescens in 10 % natrio chlorido/5 % glucoso, 0.3 M litio chlorido/liquido YM et 0.5 M litio chlorido/liquido YM. Maxima temperatura crescentiae: 45 °C (infirme). Materia amyloidea iodophila non-formantur. Ureum non hydrolysat. Diazonium caeruleum B non respondens. Typus: isolatus ex solis, JHL^T, depositus in collectione China General Microbiological Culture Collection Center, Academia Sinica (CGMCC 2.3681^T=CBS 10845^T).

Description of *Candida mengyuniae* He & Chen sp. nov.

Candida mengyuniae (meng.yun'i.ae. N.L. gen. fem. n. *mengyuniae* to honour Meng-Yun Liu, a respected Chinese microbiologist, for her contributions to the development of Chinese microbiology).

After 3 days growth at 28 °C in YPD broth, cells are ellipsoidal to elongate (2.5–5.0 × 3.0–7.2 µm), appearing singly, in pairs or in groups (Fig. 2). Budding is multilateral. Sediment is present. A fragrant ester-like odour is present. After 3 days growth at 28 °C on YPD agar medium, the streak culture is butyrous, white to cream, smooth, somewhat dull and convex, with an entire to slightly undulating margin. After 7 days Dalmau plate culture on cornmeal agar at 25 °C, pseudohyphae are formed. Ascospores are not observed. Glucose, sucrose, raffinose (weak), stachyose, D-mannitol and D-arabinol are fermented, whereas D-galactose, maltose, trehalose, melibiose, cellobiose, melezitose, inulin and starch are not. Glucose, D-galactose, D-xylose, sucrose, methyl α-D-glucoside, melibiose, raffinose, inulin, glycerol, D-glucitol, D-mannitol and citric acid are assimilated as sole carbon sources, whereas D-glucosamine, D-ribose, L-arabinose, D-arabinose, L-rhamnose, maltose, cellobiose, salicin, arbutin,

Fig. 2. Micrograph of vegetative cells of *Candida mengyuniae* sp. nov. JHL^T grown on YPD agar for 3 days at 28 °C. Bar, 10 µm.

melezitose, starch, erythritol, ribitol, xylitol, inositol and gentiobiose are not assimilated. Trehalose is assimilated. Ammonium sulfate, potassium nitrate, sodium nitrite and L-lysine are assimilated as sole nitrogen sources. Growth in vitamin-free medium is positive. Growth on 50 % glucose/yeast extract is negative. Growth in 10 % NaCl plus 5 % glucose, in 0.3 M LiCl/YM broth and in 0.5 M LiCl/YM broth is positive. Maximum growth temperature is 45 °C. Starch-like compounds are not produced. Urease activity is negative. The diazonium blue B reaction is negative.

The type strain, JHL^T (=CGMCC 2.3681^T=CBS 10845^T), was isolated from sulfonylurea-contaminated soil collected in Jiangsu Province, China, in September 2006.

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References

- Bai, F.-Y., Takashima, M. & Nakase, T. (2001). Phylogenetic analysis of strains originally assigned to *Bullera variabilis*: descriptions of *Bullera pseudohuianaensis* sp. nov., *Bullera komagatae* sp. nov. and *Bullera pseudoschimicola* sp. nov. *Int J Syst Evol Microbiol* **51**, 2177–2187.
- Bai, F.-Y., Zhao, J.-H., Takashima, M., Jia, J.-H., Boekhout, T. & Nakase, T. (2002). Reclassification of the *Sporobolomyces roseus* and *Sporidiobolus pararoseus* complexes, with the description of *Sporobolomyces phaffii* sp. nov. *Int J Syst Evol Microbiol* **52**, 2309–2314.
- Blair, A. M. & Martin, T. D. (1988). A review of the activity, fate and mode of action of sulfonylurea herbicides. *Pestic Sci* **22**, 195–219.
- Brown, H. M. (1990). Mode of action, crop selectivity and soil relations of the sulfonylurea herbicides. *Pestic Sci* **29**, 263–281.

- Fell, J. W., Boekhout, T., Fonseca, A., Scorzetti, G. & Statzell-Tallman, A. (2000). Biodiversity and systematics of basidiomycetous yeasts as determined by large-subunit rDNA D1/D2 domain sequence analysis. *Int J Syst Evol Microbiol* **50**, 1351–1371.
- Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.
- Hill, C. M. & Duggleby, R. G. (1998). Mutagenesis of *Escherichia coli* acetohydroxyacid synthase isoenzyme II and characterization of three herbicide-insensitive forms. *Biochem J* **335**, 653–661.
- James, S. A., Collins, M. D. & Roberts, I. N. (1996). Use of an rRNA internal transcribed spacer region to distinguish phylogenetically closely related species of the genera *Zygosaccharomyces* and *Torulasporea*. *Int J Syst Bacteriol* **46**, 189–194.
- Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* **16**, 111–120.
- Kurtzman, C. P. (1998a). *Williopsis* Zender. In *The Yeasts, a Taxonomic Study*, 4th edn, pp. 413–419. Edited by C. P. Kurtzman & J. W. Fell. Amsterdam: Elsevier.
- Kurtzman, C. P. (1998b). Discussion of teleomorphic and anamorphic ascomycetous yeasts and a key to genera. In *The Yeasts, a Taxonomic Study*, 4th edn, pp. 111–121. Edited by C. P. Kurtzman & J. W. Fell. Amsterdam: Elsevier.
- Kurtzman, C. P. & Robnett, C. J. (1997). Identification of clinically important ascomycetous yeasts based on nucleotide divergence in the 5' end of the large-subunit (26S) ribosomal DNA gene. *J Clin Microbiol* **35**, 1216–1223.
- Kurtzman, C. P. & Robnett, C. J. (1998). Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences. *Antonie van Leeuwenhoek* **73**, 331–371.
- Kurtzman, C. P. & Robnett, C. J. (2003). Phylogenetic relationships among yeasts of the 'Saccharomyces complex' determined from multigene sequence analyses. *FEMS Yeast Res* **3**, 417–432.
- Lu, H.-Z., Jia, J.-H., Wang, Q.-M. & Bai, F.-Y. (2004). *Candida asparagi* sp. nov., *Candida diospyri* sp. nov. and *Candida qinlingensis* sp. nov., novel anamorphic, ascomycetous yeast species. *Int J Syst Evol Microbiol* **54**, 1409–1414.
- Makimura, K., Murayama, S. Y. & Yamaguchi, H. (1994). Detection of a wide range of medically important fungi by the polymerase chain reaction. *J Med Microbiol* **40**, 358–364.
- Meyer, S. A., Payne, R. W. & Yarrow, D. (1998). *Candida* Berkhout. In *The Yeasts, a Taxonomic Study*, 4th edn, pp. 454–573. Edited by C. P. Kurtzman & J. W. Fell. Amsterdam: Elsevier.
- Ott, K. H., Kwagh, J. G., Stockton, G. W., Sidorov, V. & Kakefuda, G. (1996). Rational molecular design and genetic engineering of herbicide resistant crops by structure modeling and site-directed mutagenesis of acetohydroxyacid synthase. *J Mol Biol* **263**, 359–368.
- Pang, S. S., Guddat, L. W. & Duggleby, R. G. (2003). Molecular basis of sulfonylurea herbicide inhibition of acetohydroxyacid synthase. *J Biol Chem* **278**, 7639–7644.
- Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Scorzetti, G., Fell, J. W., Fonseca, A. & Statzell-Tallman, A. (2002). Systematics of basidiomycetous yeasts: a comparison of large subunit D1/D2 and internal transcribed spacer rDNA regions. *FEMS Yeast Res* **2**, 495–517.
- Sterflinger, K. & Prillinger, H. (2001). Molecular taxonomy and biodiversity of rock fungal communities in an urban environment (Vienna, Austria). *Antonie van Leeuwenhoek* **80**, 275–286.
- Sugita, T., Nishikawa, A., Ikeda, R. & Shinoda, T. (1999). Identification of medically relevant *Trichosporon* species based on sequences of internal transcribed spacer regions and construction of a database for *Trichosporon* identification. *J Clin Microbiol* **37**, 1985–1993.
- Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.
- Wickerham, L. J. (1951). *Taxonomy of Yeasts. Technical Bulletin 1029*. Washington, DC: USDA.
- Yarrow, D. (1998). Methods for the isolation, maintenance and identification of yeasts. In *The Yeasts, a Taxonomic Study*, 4th edn, pp. 77–100. Edited by C. P. Kurtzman & J. W. Fell. Amsterdam: Elsevier.