Biological Control 125 (2018) 15–19

Contents lists available at ScienceDirect

Biological Control

journal homepage: www.elsevier.com/locate/ybcon

Metschnikowia citriensis sp. nov., a novel yeast species isolated from leaves with potential for biocontrol of postharvest fruit rot

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ARTICLE INFO

Keywords:
Yeast
Biocontrol potential
Metschnikowia citriensis sp. nov.
Novel species

ABSTRACT

Two strains representing a yeast species of *Metschnikowia* were isolated from the surface of citrus leaves collected in Chongqing Province, China. Preliminary tests showed that this species has a biocontrol potential for inhibiting citrus green mold. This yeast species is most closely related to *Metschnikowia koreensis* based on the phylogenetic tree derived from neighbour-joining analysis of 26S rRNA gene D1/D2 domain. However, *M. koreensis* differs from this species by seven (1.5%) substitutions in the 26S rRNA gene D1/D2 domain and by > 100 (> 25%) mismatches in the internal transcribed spacer (ITS) region. The name *Metschnikowia citriensis* sp. nov. (type strain FL01^T = CICC 33213^T = KCTC 27796^T, strain FL02 = CICC 33214) is proposed for the novel species because of morphological, physiological and biochemical characteristics and the sequence comparisons of the 26S rRNA gene D1/D2 domain and ITS region.

1. Introduction

Yeasts colonize a wide range of environmental and human ecosystems because of their ability to grow and survive in variable and stressful conditions (Walker, 2011). They can easily reproduce in large quantities due to their ability to proliferate on inexpensive substrates (Spadaro et al., 2010). Over the past thirty years, biological control of postharvest pathogens based on naturally occurring microorganisms have been the most studied (Droby et al., 2009; Sharma et al., 2009; Spadaro and Gullino, 2004). Impressive progress has been made in the development, registration and commercialization of biocontrol products. Several products are available on the market, including Yieldplus™ (based on *Cryptococcus albidus*) (Janisiewicz and Korsten, 2002), Aspire™ (based on *Candida oleophila*) (Liu et al., 2013), Biosave™ (*Pseudomonas syringae* van Hall) (Janisiewicz and Peterson, 2004), Candifruit™ (a commercial formulation of *Candida sake*), BoniProtect™ (based on two antagonistic strains of *Aureobasidium pullulans*) (Spadaro and Droby, 2016) and Shemer™ (based on the yeast *Metschnikowia fructicola*) (Droby et al., 2009). Shemer™ was initially registered for both pre- and postharvest application on various fruits and vegetables (such as citrus fruit, grapes, peppers, etc.) in Israel; it was later acquired by Bayer CropScience (Germany) and recently sublicensed to Koppert (Netherlands) (Spadaro and Droby, 2016). *Metschnikowia* is an important genus of the family *Metschnikowiaceae*. Some other species of this genus have also been well exploited and widely used, including *M. reukafii* in the enzyme industry (Li et al., 2009). Additionally, *M. pulcherrima*, *M. fructicola* and *M. andauensis* have been used as postharvest biocontrol agents (Spadaro and Gullino, 2004; Kurtzman and Droby, 2001; Manso and Nunes, 2011).

Up to today, the genus *Metschnikowia* has approximately 40 species currently accepted and described (Kurtzman et al., 2011), however, the taxonomy of the yeasts has not been fully studied. Several novel yeast species were recently accepted and described, e.g., *Metschnikowia persici* (Wang et al., 2017).

The variety of the *Metschnikowia* species isolated from the surfaces of fruits and leaves demonstrated a genetic diversification in this genus (Kurtzman and Droby, 2001; Péter et al., 2005; Xue et al., 2006). In our previous research, we identified two yeast strains of *Metschnikowia* sp. (strain FL01 and strain FL02), which were isolated from the surfaces of citrus leaves in China and were highly effective in the biocontrol of storage rot of citrus induced by *Penicillium digitatum* (Liu et al., 2017).

The determination of the nucleotide sequence of the D1/D2 domain on the 26S rDNA and of the internal transcribed spacer (ITS) region of the strains together with a comparison using a database of sequences from all currently recognized ascomycetous yeasts confirmed that two strains represent novel species (Kurtzman and Robnett, 1998; White et al., 1990, and subsequent entries in GenBank). The analysis showed the new taxon to be a sister species of *M. koreensis*. Therefore, this study

Abbreviations: ITS, internal transcribed spacer; YEPD, yeast extract peptone dextrose

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https://doi.org/10.1016/j.biocontrol.2018.05.018
Received 11 March 2018; Received in revised form 21 May 2018; Accepted 24 May 2018
Available online 25 May 2018

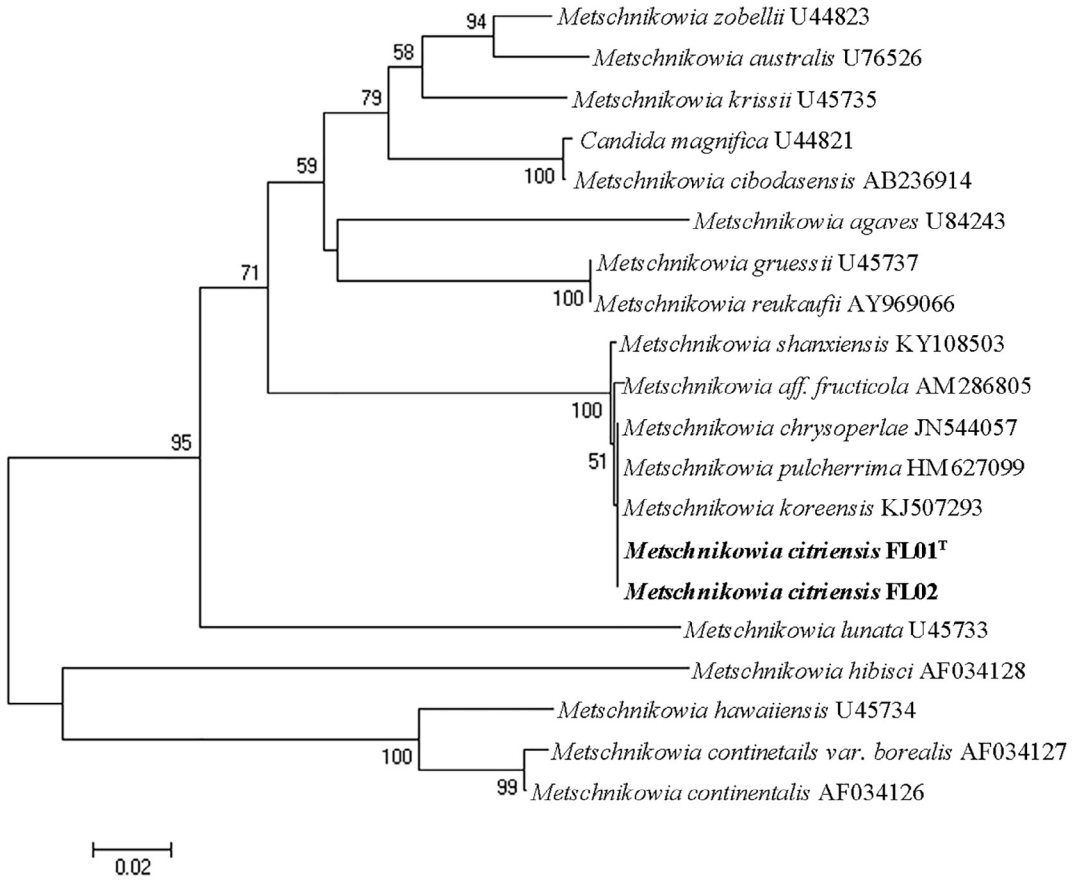

Fig. 1. Phylogenetic tree made from neighbor-joining analysis of 26S rRNA gene D1/D2 domain sequences, depicting the relationships of the novel *Metschnikowia* species with closely related taxa. Branch lengths are proportional to nucleotide differences as indicated on the bar. Numbers given at nodes are the percentage of frequencies (> 50%) with which a given branch appeared in 1000 bootstrap replicates. Reference sequences were retrieved from GenBank under the accession numbers indicated.

was aimed at describing the new species of *Metschnikowia* and discussing the biology and phylogenetic relationships of the yeasts.

2. Materials and methods

2.1. Yeast isolation and examination of taxonomic characteristics

Citrus leaves [*Citrus sinensis* (L.) Osbeck cv. Jincheng 447#] used in this study were collected from Fengjiacao orchard in Chongqing, China. Yeast strains were isolated from the surfaces of citrus leaves by the method of Nally et al. (2012) with some modifications. The isolates were purified by streaking and maintained on yeast extract peptone dextrose (YEPD) agar (5 g L^{-1} yeast extract, 8 g L^{-1} peptone, 10 g L^{-1} dextrose, 20 g L^{-1} agar, pH 5.6) at 4 °C.

The morphological, physiological and biochemical characteristics of the yeast strains were examined according to established methods as described by Yarrow (1998). Photomicrographs were made using an Olympus BX43 microscope equipped with a DP26 digital camera. The yeasts were incubated individually or mixed in pairs on corn meal agar, V8 and diluted V8 agar (1:2, 1:9, 1:19, 1:29) at 17 °C for up to 8 weeks to observe ascospores in cultures. Mycelium formation was investigated on corn meal agar in slide culture at 25 °C for up to 1 month. Growth at 35 °C and 37 °C were assessed by cultivation in YM agar. Carbon assimilation tests were conducted in liquid medium at 17 °C for 1 month, and the results were recorded weekly. Assimilation of nitrogen compounds was investigated on solid media with starved inocula (Nakase and Suzuki, 1986). The fatty acids were analyzed by using GC/MS (Kuykendall et al., 1988), according to the standard MIDI (Microbial Identification) system (Sasser, 1990). DNA-DNA hybridization and the DNA G + C content (mol%) were determined by the Identification Service, China Center of Industrial Culture Collection (CICC), Beijing, China.

2.2. Phylogenetic analysis

Nuclear DNA was extracted as described previously (Liu et al., 2017). The D1/D2 domain of nuclear 26S rDNA and the ITS (including 5.8S rDNA) region were amplified using the primer NL1 and NL4 (Kurtzman and Robnett, 1998) and ITS1 and ITS4 (Scorzetti et al., 2002), respectively. Purified PCR products were ligated into the pMD™19-T vector (Takara) and the recombined plasmid was transformed into *Escherichia coli* TOP10 according to the manufacturer's instructions. Then the cloned DNA fragments were sequenced by Sangon Biotech (Shanghai Co., Ltd.). The GenBank accession numbers for the D1/D2 sequences of strains FL01^T and FL02 are MF538699 and MF538701, and ITS sequences are MF538700 and MF538702, respectively. The sequences were compared pairwise using a BLAST search (Altschul et al., 1997) and aligned via the program CLUSTAL_X (Thompson et al., 1997). A phylogenetic tree of 26S rRNA gene D1/D2 domain sequences was constructed from evolutionary distance data calculated with the neighbor-joining method (Saitou and Nei, 1987) using Kimura's two-parameter distance measure (Kimura, 1980) by MEGA software version 7.0. Confidence levels for the individual branches of the phylogenetic tree were estimated from bootstrap analysis (1000 replications). Reference sequences were retrieved from GenBank under the accession numbers given in Fig. 1.