Candida cabralensis sp. nov., a new yeast species isolated from the Spanish traditional blue-veined Cabrales cheese

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Abstract

Three yeast strains, 1AD8, 3AD15 and 3AD23, belonging to a previously unknown yeast species were isolated from two independent batches of the Spanish blue-veined Cabrales cheese, a traditional cheese manufactured without the addition of starter and mould cultures. Physiological characterisation revealed that the unknown yeast is not fermentative and does not assimilate lactose; rather it assimilates DL-lactic acid and ethanol, major end-products from the lactic acid bacteria metabolism in cheese. The new yeast is anamorphic. The phylogenetic tree reconstruction based on nucleotide sequence comparison of the D1/D2 region of the 26S rRNA gene showed that *Pichia terricola* and *Pichia fermentans* are the closest relatives to the unknown species. The name *Candida cabralensis* sp. nov. is proposed, and the isolate 1AD8 (=CECT 13027^T, =CBS 11679^T) is the type strain of this novel taxon.

Introduction

Yeasts are common contaminants of milk and dairy products and contribute to food spoilage and even food poisoning (Pitt and Hocking, 1997). However, specific yeast species are essential for the typical characteristics of certain products, such as fermented milks (kefir, koumis, viili and longfil) and cheeses of the Brie and Camembert varieties (Roostita and Fleet, 1996; Gripon, 1999). They are also major constituents of the microbiota of many other types of cheese, including blue-veined varieties (Beresford et al., 2001; Wouters et al., 2002). Yeasts can interact with starter and non-starter lactic acid bacteria components and also with *Penicillium roqueforti* strains, promoting or inhibiting their growth (Addis et al., 2001; Gadaga et al., 2001). In addition, utilization of lactic acid by yeasts during ripening contributes to curd deacidification, which allows growth of

secondary floras in cheese (Ferreira and Viljoen, 2003). Yeasts may also have a direct role in the final organoleptical and rheological properties of cheeses through their proteolytic and lipolytic activities (Bockelmann, 1999; Ferreira and Viljoen, 2003).

Cabrales is the most famous of the traditional Spanish blue-veined cheeses and was awarded with a Protection of Designation of Origin (PDO) label in 1989. Traditional manufacture of Cabrales cheese involves curdling mixtures of evening and morning milks at 28–30ºC with a farm-made kid rennet extract, and neither starter cultures nor *P. roqueforti* spores are added to the milk (Flórez et al., 2006). Acidification and ripening relies on growth and activity of indigenous microorganisms from the milk, the rennet extract and the manufacturing and ripening environments. After a short period of drying, maturation takes place in natural caves within the area of manufacture at a nearly constant temperature (ranging from 9 to 12ºC) and humidity (≈ 95%). In these conditions, *P. roqueforti* develops into the cheese matrix, providing the final product with its original appearance and sensory characteristics. Though moderate to large differences in counts of total and indicator microbial populations are observed between batches, Cabrales is a complex microbial habitat in which many types of bacteria, yeasts and moulds interact and evolve throughout the manufacturing and ripening processes (Flórez et al., 2006).

The microbiota of Cabrales cheese was recently investigated for the presence of yeasts. Seventy-four yeast were isolated and identified (Álvarez-Martín et al., 2007). Physiological characterisation and restriction fragment length polymorphism of the ITS1-5.8S-ITS2 rDNA region indicated that three yeast isolates, 1AD8, 3AD15, and 3AD23, showed similar patterns to those of *Pichia fermentans* or *Pichia membranaefaciens*, although the isolates could not be unequivocally identified.

In this study, the complete phenotypic characterisation of strains 1AD8, 3AD15 and

3AD23 was undertaken, and a phylogenetic analysis based on the sequences of the D1/D2 domains of the 26S rRNA gene and the ITS1-5.8S-ITS2 rDNA region was performed. According to previous phylogenetic studies (Kurtzman et al., 2008), the 26S rRNA gene sequences of the new yeast isolates corresponded to a new species in the *Pichia* clade. The name *Candida cabralensis* is proposed to accommodate these three strains isolated from Cabrales cheese, *cabralen´sis*, neo-L., adj., from Cabrales, geographic area where the traditional Cabrales cheese is produced.

Yeast isolation and physiological characterisation

The three unidentified yeasts were isolated from two batches of Cabrales cheese sampled in November 2002 (strain 1AD8) and March 2003 (strains 3AD15 and 3AD23) in Tielve, a province of Asturias, Spain. Details about sample processing and isolation of yeasts are found in Álvarez-Martín et al. (2007).

Yeast morphology was examined after incubation on malt extract agar (MEA) for three days at 25ºC. Asci and ascospores production was tested by growing the isolates for up to one month at 25ºC on yeast morphology agar, acetate agar, cornmeal agar (CMA), Gorodkowa agar and 5% MEA (Yarrow, 1998). The ability to mate was tested by mixing equal amounts of young actively growing cells of the three isolates on the surface of sporulation media incubated at 15ºC and 30ºC. The plates were microscopically examined for up to 12 weeks. Morphology of the yeast cells was studied using a light microscope (Nikon, Eclipse 90i) (Figure 1). Mycelium production was tested on CMA after seven and 14 days of growth. Physiological characterisation of the strains was performed according to standard methods (Yarrow, 1998). In short, sugar assimilation tests were done on agar plates except for that of hexadecane, which was assayed in liquid medium. Assimilation of

nitrogen compounds was analysed in liquid; doubtful and false positive results were avoid by repeated inoculation of fresh tubes.

Sequencing and phylogenetic analysis

Yeast cells picked from 48 h colonies were directly used in PCR reactions as reported elsewhere (Esteve-Zarzoso et al., 1999). The D1 and D2 domains of the 26S rRNA gene and the ITS1-5.8S-ITS2 rDNA regions were amplified using the primer pairs NL1 and NL4 and ITS1 and ITS4, respectively (Kurtzman & Robnett, 2007). PCR amplification and sequencing followed the procedures of Belloch et al. (2007). Sequences were edited and assembled using MEGA version 4 (Kumar et al., 2004) and blasted against the GenBank database to retrieve sequences of the closest relatives. The sequences were included in a multiple alignment and phylogenetic trees were inferred using the neighbour-joining method (Saitou & Nei, 1987). Sequence U40085 representing the D1/D2 26S rRNA gene *Schizosaccharomyces pombe* was used as the outgroup (Kurtzman & Robnett, 1998).

Species delineation

The three strains 1AD8, 3AD15 and 3AD23 display identical D1/D2 26S rDNA gene sequences deposited in GenBank with the numbers FJ755462, FJ755463, and FJ755464, respectively. Phylogenetic analysis based on these D1/D2 26S rDNA gene sequences (Figure 2) indicated that the new yeast isolates represented a new species in the newly proposed *Pichia* clade (Kurtzman et al., 2008). The new species *Candida cabralensis* occupies an isolated position with *P. fermentans* and *P. terricola* (formerly *Issatchenkia terricola*), constituting its group of relatives. Strain 1AD8 (type of the new species) differs from *P. fermentans* NRRL Y-1619^{T} and *P. terricola* NRRL YB-4310^{T} by 73 and 62

nucleotide substitutions in the D1/D2 region respectively. The position of these three species within the *Pichia* clade is unclear because of the low bootstrap value (68%) (Kurtzman et al., 2008). Furthermore, phylogenetic reconstructions based on multigene sequence analysis did not solve the position of *P. fermentans* and *P. terricola* within the *Pichia* clade (Kurtzman et al., 2008). In the case of this group of species, the bootstrap value may increase with the isolation of more strains in its vicinity.

Sequence data and phylogenetic analysis of the ITS1-5.8S-ITS2 rDNA region confirmed the position of *C. cabralensis* as a novel isolate in the proximity of *P. fermentans* and *P. terricola*. One nucleotide difference in the sequence of the ITS1-5.8S-ITS2 rDNA region was found between the three *C. cabralensis* isolates. BLAST analysis of the ITS1-5.8S-ITS2 rDNA sequence of strain 1AD8 found the closest relatives within isolates in the species *P. terricola* (ranging from 39 to 44 nucleotide differences), whereas the strains within *P. fermentans* constituted a separate group (nucleotide differences with *C. cabralensis* ranging from 104 to 109). Physiologically, *C. cabralensis* can be clearly distinguished from its relatives by negative fermentation and positive assimilation of raffinose, inulin and melezitose. Assimilation of lactic acid and other end-products from the metabolism of lactic acid bacteria (Axelsson, 2004) argues for a tentative role of *C. cabralensis* on the ripening of Cabrales cheese.

Latin diagnosis of *Candida cabralensis* Flórez, Belloch, Álvarez-Martín, Querol et Mayo sp. nov.

In medio liquido (ME) post dies 3 ad 25ºC cellulae ovoideae aut ellipsoideae, (2–5 x 3–6 μm), singulae vel breviter catenatae, per germinationem multipolarem reproducentes.

Cultura in agaro malti (MA) post dies 5 (25ºC) convexa, butyrosa, candida, glabra. In agaro farinae Zea mays (CMA) post dies 14 pseudomycelium sparsum formantur. Ascosporae non fiunt. Fermentatio nulla. Glucosum, sucrosum, melezitosum (exigue), raffinosum, inulinum, glycerolum, ribitolum (exigue), ethanolum, acidum DL-lacticum et acidum succinicum assimilantur at non D-galactosum, L-sorbosum, cellobiosum, lactosum, maltosum, melibiosum, trehalosum, amylum solubile, D-arabinosum, L-arabinosum, D-ribosum, L-rhamnosum, D-xylosum, erythritolum, galactitolum, glucitolum, inositolum, D-mannitolum, methanolum, acidum citricum, acidum D-gluconicum, methyl-α-D-glucosidum, salicinum, D-glucosaminum hydrochlorium, N-a-D-glucosaminum, hexadecanum, saccharatum, D-glucuronatum, xylitolum nec L-arabinitolum. Lysinum, glucosaminum et ethylaminum hydrochoricum assimilantur, at non kalium nitricum, natrium nitrosum, creatininum, creatinum, imidazolum. Ureum non hydrolysatur. Materia amyloidea iodophila non formantur. Non crescit in 1% acidum aceticum nec 50% glucosum. Sine vitaminis externis supplementis crescens. Crescere potest in temperatura 30ºC at non in 37ºC.

Typus: 1AD8 (=CECT 13027^{T} =CBS 11679^{T}), ex Cabrales caseus isolata. Holotypus lyophilus conservatur in collectiones culturarum Colección Española de Cultivos Tipo (CECT) (Valentia, Hispania) et Centraalbureau voor Schimmelcultures (CSB) (Traiectum, Germaniae).

Description of *Candida cabralensis* Flórez, Belloch, Álvarez-Martín, Querol and Mayo sp. nov.

After three days at 25ºC on ME broth, cells are ovoid to ellipsoid (2–5 × 3–6 µm in

size). Cells occur singly or with one or two attached multilateral buds and in short chains. Asexual reproduction occurs by multilateral budding. After five days at 25ºC on MEA, the colony is convex, butyrous, white and smooth. After one to two weeks of growth at 25ºC on CMA, pseudohyphae are absent or only sparse rudimentary pseudohyphae occur. Sexual reproduction is negative. Fermentation is negative. The strains assimilate D-glucose, sucrose, melezitose (weakly), raffinose, inulin, glycerol, ribitol (weakly), ethanol, DL-lactic acid and succinic acid, but not D-galactose, L-sorbose, cellobiose, lactose, maltose, melibiose, trehalose, soluble starch, D- and L-arabinose, D-ribose, L-rhamnose, D-xylose, meso-erythritol, galactitol, D-glucitol, myo-inositol, D-mannitol, methanol, citric acid, D-gluconic acid, methyl-α-D-glucoside, salicin, D-glucosamine HCl, N-a-D-glucosamine, hexadecane, saccharate, D-glucuronate, xylitol and L-arabinitol. Splitting of arbutin is negative. L-lysine, glucosamine and ethylamine HCl are utilised as nitrogen sources, but not nitrate, nitrite, creatinine, creatine or imidazole. Urea is not hydrolysed. No extracellular starch is produced. No growth is observed in the presence of 1% acetic acid or 50% glucose. Growth in vitamin-free medium is positive. No growth was observed at 37ºC.

The type strain, 1AD8 (= CECT 13027^{T} =CBS 11679^{T}), isolated from Cabrales cheese were deposited in the Spanish Type Culture Collection (Valencia, Spain) and the Centraalbureau voor Schimmelcultures (CSB) (Utrecht, The Netherlands).

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FIGURE CAPTIONS

Figure 1.- Budding cells of *Candida cabralensis* 1AD8 (=CECT 13027^T) grown on ME broth for 3 days at 25ºC and 200 rpm. The image was taken with a digital Nikon Act-2U camera under phase contrast mode.

Figure 2.- Unrooted tree showing the phylogenetic relationships of type strains of neighbouring yeast species, orphan yeast strains, and *Candida cabralensis* CECT 13027^T based on the sequences of the D1 and D2 domains of their 26S rRNA gene. The sequence of the D1/D2 region from *Schizosaccharomyces pombe* NRRL Y-12796^T was used as an outgroup. In parenthesis, accession numbers of the distinct sequences. The scale bar indicates the percentage of nucleotide substitutions.

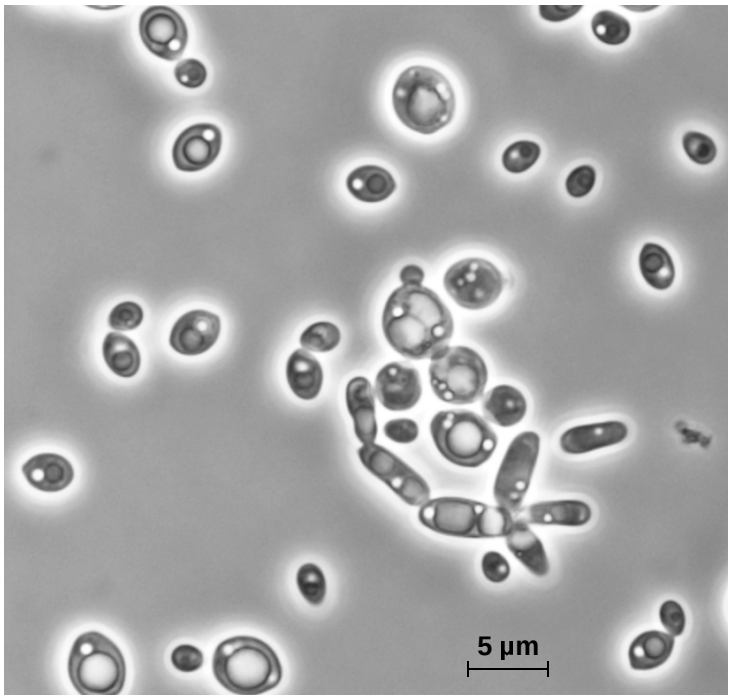

Figure 1

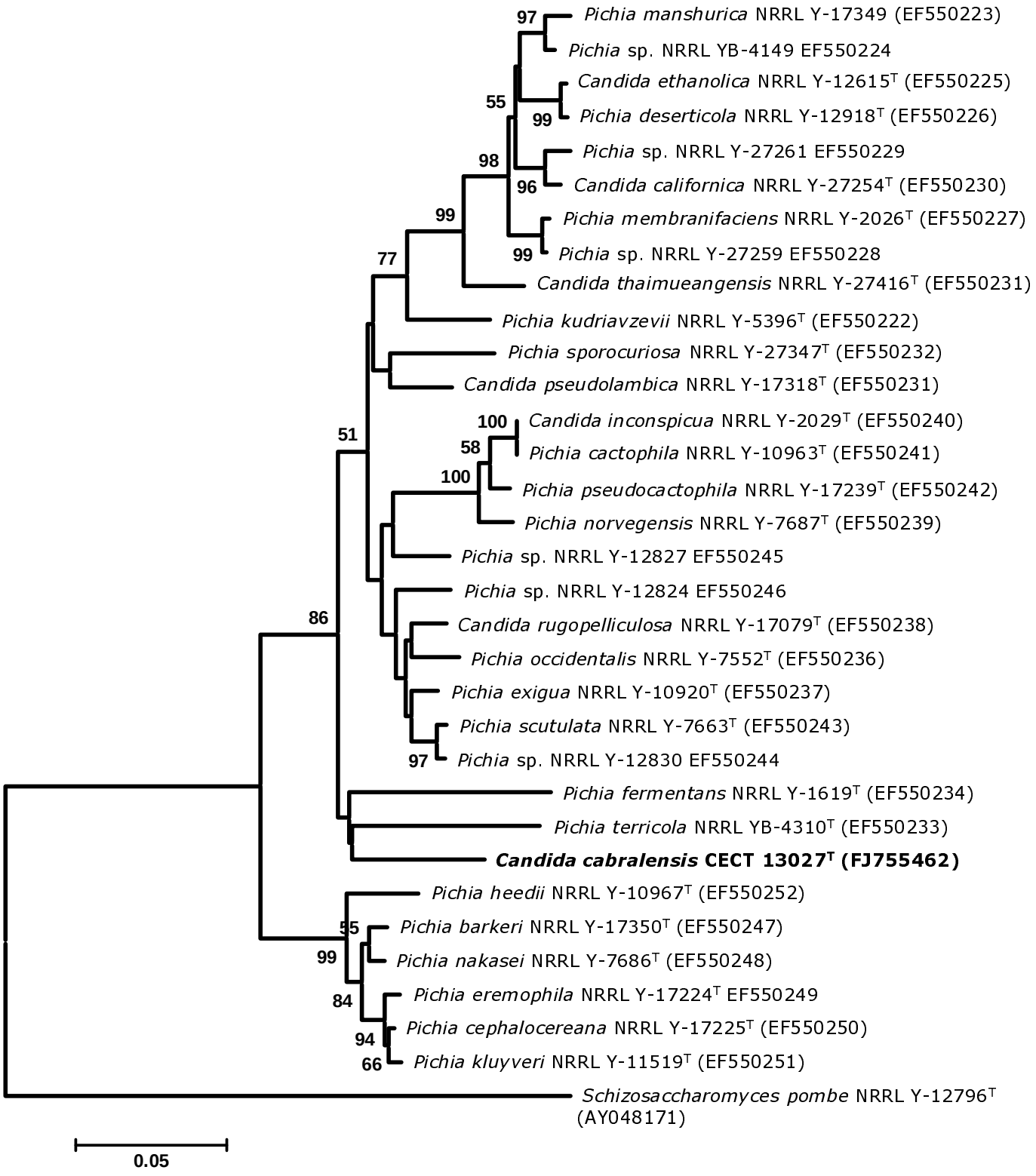
97
Pichia manshurica NRRL Y-17349 (EF550223)
Pichia sp. NRRL YB-4149 EF550224
Candida ethanolica NRRL Y-12615T (EF550225)
55
99
Pichia deserticola NRRL Y-12918T (EF550226)
Pichia sp. NRRL Y-27261 EF550229
98
96
Candida californica NRRL Y-27254T (EF550230)
99
Pichia membranifaciens NRRL Y-2026T (EF550227)
99
Pichia sp. NRRL Y-27259 EF550228
77
Candida thaimueangensis NRRL Y-27416T (EF550231)
Pichia kudriavzevii NRRL Y-5396T (EF550222)
Pichia sporocuriosa NRRL Y-27347T (EF550232)
Candida pseudolambica NRRL Y-17318T (EF550231)
100
Candida inconspicua NRRL Y-2029T (EF550240)
51
58
Pichia cactophila NRRL Y-10963T (EF550241)
100
Pichia pseudocactophila NRRL Y-17239T (EF550242)
Pichia norvegensis NRRL Y-7687T (EF550239)
Pichia sp. NRRL Y-12827 EF550245
Pichia sp. NRRL Y-12824 EF550246
86
Candida rugopelliculosa NRRL Y-17079T (EF550238)
Pichia occidentalis NRRL Y-7552T (EF550236)
Pichia exigua NRRL Y-10920T (EF550237)
Pichia scutulata NRRL Y-7663T (EF550243)
97
Pichia sp. NRRL Y-12830 EF550244
Pichia fermentans NRRL Y-1619T (EF550234)
Pichia terricola NRRL YB-4310T (EF550233)
Candida cabralensis CECT 13027T (FJ755462)
Pichia heedii NRRL Y-10967T (EF550252)
55
Pichia barkeri NRRL Y-17350T (EF550247)
99
Pichia nakasei NRRL Y-7686T (EF550248)
84
Pichia eremophila NRRL Y-17224T EF550249
94
Pichia cephalocereana NRRL Y-17225T (EF550250)
66
Pichia kluyveri NRRL Y-11519T (EF550251)
Schizosaccharomyces pombe NRRL Y-12796T (AY048171)
0.05

Figure 2