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

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Three yeast strains, 1AD8^T, 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 characterization revealed that the unknown yeast is not fermentative and does not assimilate lactose; rather it assimilates DL-lactic acid and ethanol, major end products of lactic acid bacteria metabolism in cheese. The novel yeast is anamorphic. 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 of the unknown species. The name *Candida cabralensis* sp. nov. is proposed, and the isolate 1AD8^T (=CECT 13027^T =CBS 11679^T) is the type strain of this novel taxon.

Yeasts are common contaminants of milk and dairy products and contribute to food spoilage and even food poisoning (Pitt & 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 & 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 & 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 & 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 28–30 °C 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 micro-organisms 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 12 °C) and humidity (approx. 95 %). Under these conditions, *P. roqueforti* develops into the cheese matrix, providing the final product with its unique 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 strains were isolated and identified (Álvarez-Martín *et al.*, 2007). Physiological characterization and restriction fragment length polymorphism of the ITS1–5.8S–ITS2 rDNA region indicated that three yeast isolates, 1AD8^T, 3AD15 and 3AD23, showed similar patterns to those of *Pichia fermentans* or *Pichia membranaefaciens*, although the isolates could not be unequivocally identified.

The GenBank/EMBL/DDBJ accession numbers for the D1/D2 26S rRNA gene sequences of strains 1AD8, 3AD15 and 3AD23 are FJ755462, FJ755463 and FJ755464, respectively.

In this study, the complete phenotypic characterization 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 novel yeast isolates corresponded to a novel species in the *Pichia* clade. The name *Candida cabralensis* sp. nov. is proposed to accommodate these three strains isolated from Cabrales cheese.

Yeast isolation and physiological characterization

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

Yeast morphology was examined after incubation on malt extract agar (MEA) for three days at 25 °C. Asci and ascospore production was tested by growing the isolates for up to one month at 25 °C on yeast morphology agar, acetate agar, cornmeal agar (CMA), Gorodkova 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 15 °C and 30 °C. The plates were microscopically examined for up to 12 weeks. Morphology of the yeast cells was studied using a light microscope (Nikon, Eclipse 90i) (Fig. 1). Mycelium production was tested on CMA after seven and 14 days of growth. Physiological characterization of the strains was performed according to standard methods (Yarrow, 1998). In short, sugar assimilation tests were carried out on agar plates except for that

Fig. 1. Budding cells of *Candida cabralensis* sp. nov. 1AD8^T grown on ME broth for 3 days at 25 °C and 200 r.p.m. The image was taken with a digital Nikon Act-2U camera under phase-contrast mode.

of hexadecane, which was assayed in liquid medium. Assimilation of nitrogen compounds was analysed in liquid medium; doubtful and false-positive results were avoided by repeated inoculation of fresh tubes.

Sequencing and phylogenetic analysis

Yeast cells picked from colonies grown for 48 h were directly used for PCR as reported previously (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 by 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 by using the neighbour-joining method (Saitou & Nei, 1987). Sequence U40085 representing the D1/D2 26S rRNA gene from *Schizosaccharomyces pombe* was used as the outgroup (Kurtzman & Robnett, 1998).

Species delineation

The three strains, 1AD8^T, 3AD15 and 3AD23, display identical D1/D2 26S rRNA gene sequences. Phylogenetic analysis based on these D1/D2 26S rRNA gene sequences (Fig. 2) indicated that the novel yeast isolates represent a novel species in the newly proposed *Pichia* clade (Kurtzman *et al.*, 2008). The novel species *Candida cabralensis* occupies an isolated position, with *Pichia terricola* (formerly *Issatchenkia terricola*) and *P. fermentans* 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 nt 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 multi-gene sequence analysis did not resolve 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 the vicinity.

Sequence data and phylogenetic analysis of the ITS1–5.8S–ITS2 rDNA region confirmed the position of *C. cabralensis* as a novel species 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 among isolates of the species *P. terricola* (ranging from 39 to 44 nt differences), whereas the strains representing *P. fermentans* constituted a separate group (nucleotide differences from *C. cabralensis* ranging from 104 to 109).

Fig. 2. Unrooted tree showing the phylogenetic relationships of type strains of neighbouring yeast species, orphan yeast strains and *Candida cabralensis* sp. nov. 1AD8^T based on the sequences of the D1 and D2 domains of the 26S rRNA gene. The sequence of the D1/D2 region from *Schizosaccharomyces pombe* NRRL Y-12796^T was used as an outgroup. Accession numbers are shown in parentheses. Bar, percentage of nucleotide substitutions.

Physiologically, *C. cabralensis* sp. nov. 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* in 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* 25 °C *cellulae ovoideae aut ellipsoideae*, (2–5 × 3–6 µm), *singulae vel breviter catenatae, per germinationem multipolarem reproducentes*. *Cultura in agar malti* (MA) *post dies* 5 (25 °C) *convexa, butyrosa, candida, glabra*. *In agar farinae Zea mays* (CMA) *post dies* 14 *pseudomycelium sparsum formantur*. *Ascosporae non fiunt*. *Fermentatio nulla*. *Glucosum, sucrosum, melezitosum (exigue), raffinolum, inulinum, glycerolum, ribitolum (exigue), ethanolum, acidum DL-lacticum et acidum succinicum assimulantur at non D-galactosum, L-sorbozum, cellobiosum, lactosum, maltosum, melibiosum, trehalosum, amyllum 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-acetyl-D-glucosaminum, hexadecanum, saccharatum, D-glucuronatum, xylitolum nec L-arabinitolum. Lysinum, glucosaminum et ethylaminum hydrochoricum assimulantur, at non kalium nitricum, natrium nitrosum, creatininum, creatinum, imidazolium. 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 30 °C at non in 37 °C.

Typus 1AD8^T (=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.

Candida cabralensis (ca.bra.len'sis. N.L. adj. *cabralensis* from Cabrales, the geographical area where the traditional Cabrales cheese is produced).

After three days at 25 °C 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 25 °C on MEA, colonies are convex, butyrous, white and smooth. After one to two weeks of growth at 25 °C on CMA, pseudohyphae are absent or only sparse rudimentary pseudohyphae occur. Sexual reproduction is negative. Fermentation is negative. Assimilates 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- or L-arabinose, D-ribose, L-rhamnose, D-xylose, *meso*-erythritol, galactitol, D-sorbitol, *myo*-inositol, D-mannitol, methanol, citric acid, D-gluconic acid, methyl α -D-glucoside, salicin, D-glucosamine hydrochloride, N-acetyl-D-glucosamine, hexadecane, saccharate, D-glucuronate, xylitol or L-arabinitol. Splitting of arbutin is negative. L-Lysine, glucosamine and ethylamine hydrochloride are utilized 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 is observed at 37 °C.

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

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