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Taxonomic and phenotypic characterization of yeasts isolated from worldwide cold rock-associated habitats

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ABSTRACT

Yeast strains isolated from rock samples collected from worldwide cold regions were identified by sequence analysis of the D1/D2 domains of the 26S rDNA gene and the ITS region followed by molecular phylogeny. Over 77 % of yeasts isolates were Basidiomycota. *Cryptococcus* (orders Filobasidiales and Tremellales) and *Rhodotorula* (order Cystobasidiales) were the most frequent genera. About 40 % of yeast isolates belonged to undescribed species. Almost all isolates were psychrotolerant. Urease and esterase were the most widely extracellular enzymatic activity at 4 °C and 20 °C. None of the strains exhibited extracellular protease, DNAse, cellulase, chitinase, and laccase activity.

The taxonomic and ecological significance of yeasts associated to worldwide cold rocky habitats is discussed.

Introduction

Permanently cold habitats represent an important source of Earth biodiversity: psychrophilic and psychrotolerant prokaryotic and eukaryotic microbes have been found to survive the extreme conditions of those environments (Abyzov 1993; Ma et al. 1999; Ma et al. 2000; Skidmore et al. 2000; Deming 2002; Skidmore et al. 2005; Bhatia et al. 2006; Shivaji & Prasad 2009; Buzzini et al. 2012).

Cold rock-associated ecosystems are characterized by a mixture of stressing conditions, such as cold, low water activity, oligotrophy, high solar irradiation etc, that make them inhospitable for life (Friedmann & Weed 1987; Onofri et al. 2004; Onofri et al. 2007a, b). Several studies on microorganisms

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colonizing such peculiar habitats have been carried out: filamentous and meristematic fungi, cyanobacteria, chemolitho- and chemoorganotrophic bacteria, and algae were found in microbial consortia colonizing Antarctic rocks. Fungi can be regarded as epilithic when they colonize rock surfaces, often in symbiosis with algae or cyanobacteria to form lichens. Alternatively, they inhabit the rock sub-surface as endoliths in pre-existing cracks and fissures or as cryptoendoliths in mineral pores and cavities (Ehrlich 1981; Friedmann 1982; Staley et al. 1982; Friedmann & Weed 1987; Johnstone & Vestal 1992; Friedmann et al. 1993; Urzì et al. 1995; Sterflinger 2000; Burford et al. 2003; Onofri et al. 2004; Selbmann et al. 2005; 2008).

Despite the above profusion of studies, the presence of yeasts in such extreme environments was only sporadically observed. Vishniac & Hempfling (1979a, b) isolated some yeasts from Antarctic rocks. A few years later, Vishniac (1985) described the species *Cryptococcus friedmannii* based on strains isolated from fragments of rock containing cryptoendolithic lichens. Hence, the literature published to date is almost totally restricted to Antarctica, which gives incomplete representation on the yeast diversity in these harsh ecosystems. This study reports on the results of the taxonomic and phenotypic characterization of culturable yeasts isolated from worldwide cold rock-associated habitats.

Materials and methods

Sampling sites and procedures

Rock samples have been collected from worldwide cold sites: salient information on geography and lithic nature of collected rocks are reported in Table 1. The samples were collected and processed over a period of 13 y (1996–2008). Samples of colonized rocks were aseptically removed with a sterile chisel, placed in sterilized plastic bags and preserved at −20 °C before being processed.

Yeast isolation from rock samples

Rocks from the Antarctic were aseptically fragmented and seeded on Petri dishes containing 2 % Malt Extract Agar (MEA, AppliChem GmbH, Darmstadt, Germany). Rocks collected in other environments than Antarctica were washed 15 min in sterile physiological solution added with 0.1 % Tween 20 (Sigma–Aldrich, Munich, Germany) and rinsed 4 times with physiological solution to remove potential contaminants. All rock samples were powdered and seeded both on Petri dishes containing 2 % MEA and dichloran-rose bengal agar (DRBC, Oxoid Ltd., Basingstoke, Hampshire, U.K.). Media were supplemented with chloramphenicol 100 ppm to prevent bacterial growth. All the experiments were performed in triplicate. Dishes were incubated at 10 °C. Yeast colonies grown on Petri dishes were periodically checked for several months: all yeast colonies were picked for isolation. After isolation, yeast colonies were sub-cultured on MEA slants. All yeast isolates are deposited in the Culture Collection of Fungi from Extreme Environments CCFEE of the University of Tuscia, Viterbo (Italy), and in the Industrial Yeasts Collection DBVPG of the University of Perugia (Italy) (www.agr.unipg.it/dbvpg). The CCFEE and DBVPG accession numbers are reported in Tables 1 and 2.

Molecular identification of yeasts: DNA extraction, amplification and sequencing

Yeasts were grown on MEA slants for about 1 m at 10 °C. DNA extraction used the Nucleospin Plant kit (Macherey–Nagel, Düren, Germany) following the protocol optimized for fungi. PCR reactions employed BioMix (BioLine GmbH, Luckenwalde, Germany). In each 25 μL reaction mixture, 5 pmol of each primer and 40 ng of template DNA were added. The amplification used a MyCycler™ Thermal Cycler (Bio-Rad Laboratories GmbH, Munich, Germany). The molecular studies were based on sequencing of the D1/D2 of LSU rDNA. ITS region was also sequenced for strains with ambiguous D1/D2 BLAST results. For the D1/D2 amplification the primers LR0R (http://biology.duke.edu/fungi/mycolab/primers.htm) and LR7 (Vilgalys & Hester 1990) were used and the PCR conditions were as follows: 5 min at 95 °C for a first denaturation step, a denaturation step at 95 °C for 45 s, annealing at 55 °C for 30 s and extension at 72 °C for 2 min. Cycles were repeated 35 times, with a last extension at 72 °C for 5 min. For the amplification of the rDNA ITS the primers ITS1 and ITS4 (White et al. 1990) were applied with the following PCR conditions: 3 min at 95 °C for a first denaturation step, a denaturation step at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s. Cycles were repeated 35 times, with a last extension at 72 °C for 5 min.

The amplicons were purified using Nucleospin Extract kit (Macherey–Nagel, Düren, Germany) and sequenced with the TF Big Dye Terminator 1,1 RR kit (Applied Biosystems, Foster City, CA, USA). Fragments were analyzed by Macrogen Inc. (Seoul, Korea, http://www.macrogen.com). Sequence assembly was performed with the software ChromasPro (version 1.32, Technelysium Ltd, Australia); both strands were sequenced to assure the accuracy of sequence reconstruction. The GenBank accession numbers of both rDNA D1/D2 and ITS sequences (www.ncbi.nlm.nih.gov) are shown in Table 2.

Alignment and phylogenetic trees reconstruction

D1/D2 sequences were compared in the public domain (www.ncbi.nlm.nih.gov/BLAST). Most sequences matched with the genus *Cryptococcus*: the most similar were selected and aligned with Muscle (www.ebi.ac.uk/tools/msa/muscle) implemented in Mega5 software. The final alignment, based on 638 positions including gaps, was exported and the best-fit substitution model was determined with Modeltest MrAic.pl 1.4.3 (Nylander 2004) and estimated with Phyml (Guindon & Gascuel 2003) through hierarchical likelihood ratio tests, weighting the nucleotide substitution model and model uncertainty. All 56 models implemented in Modeltest were evaluated. Phylogenetic trees were reconstructed by Maximum Likelihood (ML), using Treefinder (Jobb et al. 2004) and the resulting tree was displayed with Treeview v. 1.6.6 (Page 1996). The robustness of the phylogenetic inference was estimated with the bootstrap method (Felsenstein 1985) with 1000 pseudoreplicates generated and analyzed with Treefinder.

A selection of the most similar sequences, which matched with the genera *Rhodotorula* and *Taphrina* was