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Species richness and association with fauna

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Keywords: Yeasts - Hydrothermal vents - Fauna - 26S rRNA gene – FISH

Running title: Culturable yeasts from hydrothermal vents

Abstract

The diversity of culturable yeasts at deep-sea hydrothermal sites has been investigated and raises emerging hints regarding interactions with endemic fauna. Samples were collected during diverse oceanographic cruises at Mid-Atlantic Ridge, South Pacific Basins and East Pacific Rise. A culture collection of thirty-two isolates mostly associated with animals was performed. The phylogenetic analyses of 26S rRNA gene sequences revealed that the yeasts belonged to *Ascomycota* and *Basidiomycota* phyla with the identification of several genera: *Rhodotorula*, *Rhodosporidium*, *Candida*, *Debaryomyces*, and *Cryptococcus*. Those genera are usually isolated from deep-sea environments. To our knowledge, this is the first report of yeasts associated with deep-sea hydrothermal animals.

Introduction

Yeasts are ubiquitous microorganisms that represent a part of the microbiota in all natural ecosystems such as soils, freshwaters and marine waters from ocean surface to deep-sea. Marine yeasts are divided into *obligate* and *facultative* groups. “Obligate marine yeasts” are yeasts that have never been isolated from anywhere else than marine environment, whereas “facultative marine yeasts” are also known from terrestrial habitats (Kohlmeyer and Kohlmeyer, 1979). Based on these definitions, Kohlmeyer and Kohlmeyer (1979) have examined yeasts occurring in marine environments and have gathered a list of 176 species isolated from diverse marine habitats. Out of those, only 25 were obligate marine yeasts widely represented by the genera *Metschnikowia*, *Rhodosporidium*, *Candida* and *Torulopsis*.

The existence of 1.5 million fungal species as hypothesized by Hawksworth (2002) is a commonly used and accepted figure. If this estimate is correct, less than 5% of the fungi have been described up to now and almost exclusively from terrestrial environments. In this ecosystem, fungi are known to utilize a wide spectrum of simple and more complex organic compounds. The decomposition activities of fungi are clearly important in relation to the redistribution of elements among organisms and environmental compartments (Gadd, 2007). Bearing in mind those parameters, our hypothesis is that deep-sea and especially hydrothermal vents, which remain underexplored habitats for fungi, could be ecological niches hosting specific fungal communities.

Deep-sea hydrothermal vents are localized at sea floor spreading centers called *rifts* where seawater seeps into cracked regions caused by the presence of hot basalt and magma.

Seawater carrying dissolved minerals is then emitted from springs. Two major types of emissions have been found. Warm fluids diffuse at temperatures ranging from 6 to 23°C into seawater at 2-4°C when hot vents called black smockers emit hydrothermal fluid at 270-380°C (Munn, 2003). Thermal gradients in hydrothermal vents are so important that just a few centimeters away, the temperature can fall to 2-4°C allowing mesophilic or psychrophilic organisms as well as thermophilic and hyperthermophilic prokaryotes to grow and interact with all biotic or abiotic components of these ecosystems. Dense animal communities cluster around those hot springs. These communities are supported by the chemolithoautothrophic activities of prokaryotes (Jorgensen and Boetius, 2007).

The occurrence of fungi (filamentous fungi and yeasts) at deep-sea hydrothermal vents remains an underexplored topic. Over the last years, the interest for the diversity of microbial eukaryotes in these ecosystems emerged using PCR amplification of SSU ribosomal RNA genes and sequence analysis (Edgcomb *et al*. 2002; Lopez-Garcia *et al*., 2003; 2007). These papers revealed a scarce fungal diversity but some sequences were novel. Only two papers have specifically dealt with fungal diversity at deep-sea hydrothermal vents based on culture-dependent methods (Gadanho & Sampaio, 2005; Burgaud *et al*, 2009). Culturable yeasts affiliated to *Ascomycota* and *Basidiomycota* phyla were reported from hydrothermal waters. Some papers assessing fungal diversity at deep-sea vents were also published. Bass *et al*. (2007) reported the presence of sequences affiliated to *Debaryomyces hansenii* and novel sequences closed to *Malassezia furfur* in hydrothermal sediments. Le Calvez *et al*. (2009) reported that fungal diversity from deep-sea vent animals was widely constituted of sequences affiliated to *Chytridiomycota* and *Basidiomycota* phyla. The latter phylum was mostly represented by yeasts with, for example, the *Cryptococcus* and *Filobasidium* genera that form dense clusters.

The occurrence of yeasts in other deep-sea environments has been much more studied. Nagahama *et al*. (2001b) reported that culturable fungal diversity was dominated by ascomycetous yeasts in surface sediments in water depths exceeding 2000 meters (*Candida*, *Debaryomyces*, *Kluyveromyces*, *Saccharomyces* and *Williopsis*). Inversely, diversity was dominated by basidiomycetous yeasts on the subsurface of sediments in water depths exceeding 2000 meters and from deep-sea clams, tubeworms and mussels (*Rhodotorula*, *Sporobolomyces, Cryptococcus* and *Pseudozyma*). Recent studies have clearly demonstrated that *Cryptococcus* was the dominant genus sequenced from sediments collected at deep methane cold seeps (Takishita *et al*., 2006; 2007). Those observations are in agreement with

Bass *et al*. (2007) who suggest that yeast forms dominate fungal diversity in deep oceans. Several yeasts mostly isolated from deep-sea sediments represented new species in the *Ascomycota* or *Basidiomycota* phyla (Nagahama *et al*., 1999; 2001a; 2003a; 2003b; 2006a; 2008).

In this study, we decided to assess the presence of yeasts at deep-sea hydrothermal vents based on a culture-based approach with an emphasis on yeasts in interactions with the endemic animal fauna thriving in these extreme ecosystems. A recent paper (Gadanho and Sampaio, 2005) has dealt with the diversity of yeasts in deep-sea vent waters but, to our best knowledge, this is the first report of the culturable yeasts isolated from deep-sea animals. Those interactions with the fauna are discussed based on the cultures obtained from the samples collected during different oceanographic the cruises at Mid-Atlantic Ridge, South-West Pacific Lau Basin and East Pacific Rise.

Materials and methods

Environmental sampling

210 hydrothermal samples were collected during 6 oceanographic cruises at several dates and locations (For hydrothermal vents locations, see Tivey, 2007): (i) BIOLAU in the Lau Basin, South-west Pacific (12/05/1989–27/05/1989; 20°3.0′S, 176°7.8′W; -2620 m); (ii) DIVANAUT2 (19/06/ 1994–01/07/1994) on the MAR at Menez Gwen (37°51′N, 31°31′W; -900 m) and Lucky Strike (37°17′N, 32°16′W; -1650 m) hydrothermal sites; (iii) HERO on the EPR at Elsa site (30/09/1991–04/11/1991; 12°48′N, 103°57′W; -2630 m); (iv) MARVEL (29/08/1997–13/09/1997) on the MAR at Menez Gwen and Lucky Strike sites; (v) EXOMAR (25/07/2005–28/08/2005) on the MAR at Rainbow (36°08′N, 34°00′W, -2300 m), TAG (26°02′N, 44°54′W, -3630 m) and Lost City (30°04′N, 42°12′W, -900 m) sites; (vi) MoMARDREAM-Naut (08/07/2007–19/07/2007) on the MAR at Rainbow site. Depending on cruises, deep-sea sampling was performed using the Deep Submergence Vehicle "Nautile" or the Remote Operated Vehicle (ROV) "Victor 6000" and the N/O "Atalante" and "Pourquoi Pas?" research vessel.
The deep-sea samples were processed as described by Burgaud *et al* (2009) taking care to avoid contamination in applying strict sterile sampling conditions.

Enrichment conditions and isolation

The samples were processed directly after the Nautile or ROV recovery. The ollected samples mainly composed of deep-sea hydrothermal vent animals (*Rimicaris exoculata* and

125 *Chorocaris chacei* shrimps and *Bathymodiolus azoricus* mussels) were used to inoculate the
126 GYPS culture medium that led to the best isolation rate during a previous study (Burgaud *et*
127 *al*., 2009). This medium contained per liter: glucose (Sigma) 1 g, yeast extract (AES) 1 g,
128 peptone (AES) 1 g, starch (Fisher) 1 g, sea salts (Sigma) 30 g. This medium was
129 supplemented per litre with agar 15 g and chloramphenicol (Sigma) 500 mg, pH was also
130 adjusted to 7.5. Cultures were done aerobically at 4°C, 15°C, 25°C (ambient temperature), 35
131 and 45°C (only during EXOMAR) at atmospheric pressure until fungal strains were
132 visualized. During the MoMARDREAM-Naut cruise, some dissections were realized on
133 board on animal samples in order to investigate the yeast location.
134 Each purified strain from our collection (Table 1) has been integrated to the 'Souchothèque de
135 Bretagne' culture collection
136 (http://www.ifremer.fr/souchotheque/internet/htdocs/generique.php?pagebody=catalogue.php)
137 and are available with an accession number associated to their GenBank number.
138

139 ***Physiological characterization and statistical analysis***

140 All experiments were done in triplicate. The yeasts were grown in liquid GYPS broth media.
141 The effect of temperature on growth was determined at 5°C, 15°C, 25°C and 35°C at 30 $g.L^{-1}$
142 sea salts. The effect of salinity was analyzed modifying sea salts concentrations in media from
143 0 to 60 g.L-1 with steps of 15 g.L-1 at optimal temperature for each strain. Optical densities
144 (OD) were measured at 600 nm with Nanocolor 100D (Macherey-Nagel, Hoerdt, France) at
145 17, 22, 25 and 28 hours of growth under each condition of salinity and temperature.
146

147 ***DNA extraction and 26S rDNA sequencing***

148 DNA of each strain was extracted using FastDNA Spin Kit (MP Biomedicals, Illkirch,
149 France) specific for fungi and yeasts. Amplifications of the D1/D2 region of 26S rDNA were
150 carried out with rDNA primers ITS5 (5'-GGA AGT AAA AGT CGT AAC AAG-3'), LR6
151 (5'-CGC CAG TTC TGC TTA CC-3'), NL1 (5'-GCA TAT CAA TAA GCG GAG GAA
152 AAG-3') and NL4 (5'-GGT CCG TGT TTC AAG ACG G-3') as described by Gadanho &
153 Sampaio (2005). All PCR reactions were performed in 20 µL reaction volumes containing 19
154 µL of 1X PCR Buffer (Promega), 2 mM of MgCl2, 0.2 mM of each dNTPs (Promega), 0.6
155 µM of primers (forward and reverse), 1.25 U of Taq DNA Polymerase (Promega) and 1µL of
156 DNA. The polymerase chain reactions were performed on PTC-200 (Biorad, France). The
157 amplification consisted in an initial denaturation step at 94°C for 2 min, followed by 30

158 iterations of 15 sec at 94°C, 30 sec at 54°C, 1 min at 72°C and a final extension step of 2 min
159 at 72°C. A negative control with sterile distilled water replacing DNA was added. Two kinds
160 of amplification were generated using ITS5-NL4 and NL1-LR6 primers. The amplified DNA
161 fragments were separated by electrophoresis in 0.8% agarose (w/v) gel (Promega) in 0.5X
162 Tris-Borate-EDTA (TBE) Buffer at 90 V for 1h and stained with ethidium bromide. A
163 molecular size marker was used for reference (Lambda DNA/EcoR1+Hind III Markers,
164 Promega). The DNA banding patterns were visualized under UV transillumination and
165 picture files were generated using Gel-Doc 2000 (Biorad, France).

166 The sequencing of the D1/D2 region of the 26S rDNA was then realized using NL1 on the
167 ITS5-NL4 fragments and NL4 on the NL1-LR6 fragments. The sequences were obtained by
168 "Big Dye Terminator" technology (Applied Biosystems). This work was done at
169 "Biogenouest" sequencing facility in the "Station Biologique de Roscoff" (www.sb-
170 roscoff.fr).

171 ***Phylogenetic analyses***

172 Sequences were edited and cleaned using Sequencher v 4.8 (Gene Codes). Sequences were
173 then imported to MEGA 4.0 software (Tamura *et al.,* 2007). Each sequence was analyzed in
174 order to find GenBank sequences with close BLAST-N hits (Altschul *et al*., 1990).
175 Similarities between sequences were assessed using pairwise distance calculation with
176 MEGA 4.0. The sequences were trimmed to ensure that all sequences had the same start and
177 end-point. All the D1/D2 regions of the 26S rDNA sequences were aligned using ClustalW
178 v.1.83 (Thompson *et al*., 1994). After visual checking and manual curation, an alignment
179 composed of 62 taxa and 579 characters was analysed for the Bayesian estimation of
180 phylogeny using MrBayes v.3.1.2 software (Ronquist and Huelsenbeck, 2003). A two-million
181 generation option has been set to run the Metropolis-coupled Monte Carlo Markov Chain
182 method (*mcmc*). After generation 2 000 000, the standard deviation of split frequencies was *P*
183 = 0.005997 indicating that a convergence had occurred (P-value of < 0.05). The alignment
184 was analysed using MODELTEST v.3.7 (Posada and Crandall, 1998), in order to obtain the
185 more realistic evolutionary model used for phylogenetic analyses (GTR + G model; gamma-
186 distribution shape parameter = 0.3978). Phylogeny was then evaluated using two different
187 methods: (i) Bayesian inference with MrBayes v.3.1.2 analysis using 2 000 000 generations
188 and the *mcmc* method. The tree search included two *mcmc* searches with four chains (setting
189 default temperature for heating the chains) and a sampling frequency of 100 generations. A
190 'burnin' of 5000 (25% of the 2 000 000 generations/100 sample frequency) was set in order to
191 exclude the first 5000 trees generated. (ii) Maximum likelihood with 100 bootstrap iterations

using PHYML (Guindon *et al*., 2005) and the parameters obtained with MODELTEST v.3.7. The final phylogenetic tree topology was realized using MrBayes v.3.1.2 analysis results. Nodes in the tree show Bayesian posterior probabilities and ML bootstraps respectively.

Fluorescent probe design and evaluation

For the detection of yeasts isolated from deep-sea vent animals by FISH, we designed oligonucleotide probes using the Primrose software (http://www.bioinformatics-toolkit.org/Primrose/index.html) as described by Ashelford *et al*. (2002) using a set of high-quality, full-length rRNA sequences of probe target organisms. The PrimRose design tool permitted to produce oligonucleotide probes for the three principal clusters of our collection (Table 3). These probes exhibited no mismatches with the target organisms but exhibited mismatches with the next most similar sequences in the GenBank database proving that the designed probes were *in silico* highly specific. The target sites of newly designed probes were checked for accessibility using the prediction maps based on the 26S rRNA of *Saccharomyces cerevisiae* (Inacio *et al*., 2003). Each probe was in a relative accessible area of the 26S rRNA secondary structure (Fig S1). As it was not possible to test the probes with culture isolates that exhibited zero or one mismatch with the probes, we used an alternative method and tested the probes against all strains from our collection displaying two or more mismatches with the oligonuceotides. All newly designed probes were labelled at the 5' terminus with the fluorescent marker Cy3. All probes were synthesized by (Proligo, France) and stored in sterile distilled water at -20°C. The newly designed probes were checked under *in situ* conditions with target and non-target species. The universal probe Euk516-Fluorescein (5'-ACCAGACTTGCCCTCC-3'; Amann *et al*., 1995) and the non-Euk516-Cy3 (5'-CCTCCCGTTCAGACCA-3') probes were used as positive and negative control respectively. The average cell brightness was measured using different formamide concentrations from 0 to 80% with steps of 10%. Systematic evaluation of the signal intensities was done by recording images of independent visual fields (encompassing at least 100 cells), followed by digital image analysis using the daime software (Daims *et al*., 2006). During this step, the intensities of the image pixels analyzed enable determination of single cell fluorescence in relative units (RU).

Fluorescence In Situ Hybridization

On environmental samples. Interior branchiostegites of *Rimicaris exoculata* shrimps and byssus of *Bathymodiolus azoricus* mussels were processed for FISH analyses. Following harvest and dissections, animal subsamples were fixed with 4% paraformaldehyde solution in phosphate-buffered saline (PBS) for 3 hours at 4°C in a dark room. After fixation, tissues were washed three times with PBS and stored at -20°C in a storage buffer containing PBS and 96% ethanol (1:1).

On membrane filters. The seawater surrounding shrimps (MoMAR08, Rainbow) was sampled in 5 L sterile sampling bags using a peristaltic pump. Immediately after dives, seawater samples for *in situ* hybridizations were mixed with 3 % formaldehyde (final concentration) 2 hours at 4°C. Fixed seawater was then filtered on polycarbonate membranes 0.22 μm (Nuclepore®, 47 mm diameter; Whatman, Maidstone, Kent, UK) and rinsed with a PBS 2X - sterile seawater (v:v) buffer. Then filters were dehydrated using ethanol series (50 %, 80 % and absolute, 3 min each). Dried filters were stored at -20°C until hybridization treatments. Three membranes were treated in this study. The filtered volume was 0.8 L for membrane A, 1 L for membrane B and 1.5 L for membrane C. The filtered seawater on membranes A and B was from the same sample.

The samples (environmental samples and membrane filters) were cut in squares and paste with one drop of 0.2% low-gelling point agarose (35-40°C) on slides (Menzel-Glaser, Germany). All slides were then dipped in 0.2% agarose and air dried. Samples were then subjected to dehydratation with increasing concentrations of ethanol (50, 80, and 96%, for 3 min each). Working solutions of probes had a concentration of 30 ng of DNA per liter. The hybridization buffer, containing 0.9 M NaCl, 20 mM Tris-HCl (pH 7.2), 0.03% SDS, and 0, 10, 20, 30, 40, 50, 60, 70 and 80% formamide, and the fluorescent probe were gently mixed in a ratio of 10:1 (vol/vol) to get a final oligonucleotide concentration of 3 ng per liter. For hybridization, slides were placed in sampling tubes and incubated at 46°C in the dark for exactly 3 hours. Following hybridization, the slides were washed with prewarmed washing buffer (20 mM Tris/HCl, 5 mM EDTA (pH 8.0) and 900, 450, 215, 102, 46, 18, 5, 0,6 and 0 mM NaCl corresponding respectively to 0, 10, 20, 30, 40, 50, 60, 70 and 80% formamide stringencies) for 20 min at 48°C. Slides were rinsed with double-distilled water, air dried, DAPI stained (final concentration 1 µg/ml) and mounted with the antifading reagent Citifluor AF 2 (Citifluor, France) before observations under fluorescent microscope.

Results

Yeast isolation

Yeasts were not found in all the studied sites as shown in Table 1. No yeast was isolated from samples collected during HERO (on the East Pacific Rise at Elsa site), DIVANAUT2 and MARVEL (Menez-Gwenn and Lucky Strike) cruises or at TAG site during the EXOMAR oceanographic cruise. The hydrothermal site that yielded the highest number of isolates was clearly Rainbow (29 isolates out of 32 strains). Rainbow is also the site where the highest number of samples was processed (97/210). The yeast collection obtained from deep-sea samples raised thirty-two isolates that could be divided in pigmented yeasts (18) and non-pigmented yeasts (14). Pigmented yeasts consisted widely of red-pigmented yeasts (16), black-pigmented yeast (1) and brown-pigmented yeast (1).

Regarding yeast isolation versus type of substrate, strains were obtained mostly from hydrothermal shrimps *Rimicaris exoculata* (11), *Chorocaris chacei* (3), *Mirocaris fortunata* (1) and from hydrothermal mussels *Bathymodiolus azoricus* (7). Carbonate colonization modules deployed for 1 year near Rainbow vent yielded a few yeasts (4); sponges led to the isolation of three yeasts. Finally, seawater, gastropods and coral samples permitted to obtain one strain each (Table 1). Those results indicate that yeasts were much more associated with animals rather than mineral substrates. Statistical distribution tests have been performed in order to find out the distribution type of yeasts in hydrothermal sites. The variance to mean ratio (s2/m) was calculated for each site (Cancela da Fonseca, 1966). Values of s2/m significantly different of 1 corresponds with (s2/m) - 1 > 2(2n/(n - 1)2)1/2 and were obtained only for Rainbow site. For this hydrothermal site, an aggregate distribution was observed (s2/m=1.32) indicating that the culturable yeasts isolated were located in specific niches in this hydrothermal site (mainly shrimps and mussels).

During the MoMARDREAM-Naut cruise, dissections of body components were processed for all shrimps (branchiostegites, scaphognathites, exopodites, gills, stomach and digestive tract) and mussels (interior and external faces of shells) to investigate the localization of yeasts in deep-sea animals. For shrimps, a large majority of strains were grown from the inner side of the branchiostegites that can be divided in 3 different compartments: (a) an antero-ventral area, which was relatively clear; (b) a posterior area, which always remained light beige; (c) an antero-dorsal area with an intensely rusty coloration (for schematic views, see Zbinden *et al*., 2004; Corbari *et al,* 2008). Yeast isolates resulted from this study were all cultivated from the antero-dorsal area characterized by high amounts of minerals and a dense bacterial mat.

The yeasts were also isolated from *Bathymodiolus azoricus* (7) during the MoMADREAM-

Naut oceanographic cruise (Table 1). Most of them (6) were cultivated from external face of the mussel shells and more precisely from the byssus that is a network of filaments allowing attachment to rocks. This tangle gathers a lot of particles and organic matter in decomposition (personal observation). Only one yeast was isolated from the interior of a mussel (Mo32).

Physiological analysis

Three categories of strains were identified (Table 2) based on the definition of halotolerant and halophilic microorganisms (Margesin & Schinner, 2001; Kushner, 1978). Non halophiles are strains with maximal growth without sea salts and a decreasing growth rate with increased sea salts concentration in media. Halotolerant yeasts are strains able to grow in the absence as well as in the presence of salt. Halophiles required salt for an optimal growth. Regarding halophily, optimal salinities, optimal temperatures and OD measurement, 9 physiological groups were defined. Most of the isolated strains were non halophiles (23 strains) and halotolerant (2 strains, maximal OD at 30 g/l sea salts) growing efficiently at an optimal temperature of 25°C. Four strains had poor maximal growth at 25°C including 1 non halophile, 2 halotolerant (maximal OD at 30 and 60 g/l sea salts) and 1 halophile (maximal OD at 30 g/l sea salts). Three strains had maximal and efficient growth at 35°C, including 1 non halophile, 1 halotolerant (maximal OD at 45 g/l sea salts) and 1 halophile (maximal OD at 30 g/l sea salts).

Identification

For species identification, a sequence analysis of the D1/D2 domain of the 26S rRNA gene was done (Fig 1). A total of 12 phylotypes was found among the collection of yeasts isolated from deep-sea hydrothermal vents. Eleven phylotypes could be assigned to a known yeast species and one represents a new yeast species.

Within *Basidiomycota*, the *Sporidiobolales* order was the dominant cluster composed of 16 strains. A majority of strains (Ex2, Ex3, Ex4, Ex5, Ex6, Ex7, Ex9, Ex11, Ex12, Mo32, Mo35 and Mo37) was identified as *Rhodotorula mucilaginosa* (100% similarity). A large majority of *R. mucilaginosa* was isolated from deep-sea shrimps (14) and the others from deep-sea mussels (2). As member of the *Sporidiobolales* order, isolates affiliated to *Rhodosporidium diobovatum* were also isolated (Mo24, Mo33 and Mo38) with 100% similarity. These 3

strains were isolated respectively from *Rimicaris exoculata* exuviae in decomposition on smocker rocks, *Bathymodiolus azoricus* and a sponge. One strain isolated from *R. exoculata* was identified as *Sporobolomyces roseus* based on 26S rRNA genes (Mo22) with 100% similarity with the reference strain. Four strains (Mo26, Mo27, Mo28 and Mo29) were affiliated to the *Filobasidiales* order and identified as *Cryptococcus uzbekistanensis* (100% similarity). These four strains were all isolated from a carbonate colonization module. Finally, one isolate (Mo36) from *B. azoricus* mussel was identified as *Leucosporidium scottii* in the *Leucosporidiales* order.

The *Ascomycota* phylum gathered 9 strains belonging to the *Saccharomycetales* order. Within this order, 4 strains (Mo20, Mo21, Mo40 and Bio2) isolated respectively from *R. exoculata*, *Mirocaris fortunata*, a deep-sea coral and the gills of the gastropod *Ifremeria nautilei* were identified as *Debaryomyces hansenii* (100% similarity). *Candida atlantica* isolates were found in *R. exoculata* exuviae in decomposition (Mo25) and *B. azoricus* (Mo31). One strain isolated from a deep-sea sponge (Ex15) was identified as *Pichia guilliermondii* (100% similarity). Finally, among the *Saccharomycetales* order, one strain was identified as *Candida viswanathii* (Bio1) with 100% similarity. One halophilic strain (Mo39) isolated from a deep-sea coral represents a new species in the *Candida* genus and thus was identified as *Candida* sp. This strain has 95% similarity with the reference sequence of *Candida atmosphaerica* (23 mismatches on 505 bp). Mo30 isolated from *Bathymodiolus azoricus* was identified as *Phaeotheca triangularis* (mitosporic *Ascomycota*) with 100% similarity. In the *Dothideales* order, one strain (Mo34) isolated from *Bathymodiolus azoricus* was identified as *Hortaea werneckii* with 99.98% similarity (one mismatch on 560bp).

Sequencing of the 26S rRNA genes indicated the presence of *Ascomycota* and *Basidiomycota* in our culture collection. In term of quantity, the phylum *Basidiomycota* (21) was two times higher than the *Ascomycota* (11). In term of species richness, ascomycetous yeasts belonged to 7 different clusters while basidiomycetous yeasts belonged to 5 clusters.

Fluorescence *in situ* hybridizations

We processed numerous assays to detect fungi on deep-sea hydrothermal vent animal samples using different existing fluorescent probes from different databases. The Euk516-Cy3 probe gave positive results on pure cultures but strong background fluorescence on hydrothermal samples led to the renouncement of its use. The probe MY1574 targeting *Eumycota* organisms (Baschien *et al*., 2008) showed very weak fluorescence on pure cultures. Thus, we

decided to design our own probes (Table 3) based on our culture collection that was divided in 3 main clusters: **MitoFilo** (*Cryptococcus* / Mitosporic *Filobasidiales* order), **MitoSporidio** (*Rhodotorula*, *Rhodosporidium* / Mitosporic *Sporidiobolales* order) and **Sacch** (*Debaryomyces*, *Pichia* / *Saccharomycetales* order). The probes designed revealed a strong specificity for the target organisms. The optimal conditions for the *in situ* hybridization protocol use stringent conditions of 20% formamide (Fig S1).

Our aim was to check the applicability of the FISH method to the *in situ* detection of yeasts in deep-sea hydrothermal fauna samples. Hydrothermal body components of endemic shrimps (*Rimicaris exoculata*) and mussels (*Bathymodiolus azoricus*) were fixed for FISH experiments directly after dissection. The pieces of shrimps and mussels that gave the higher number of fungi isolation (interior branchiostegites of shrimps and byssus of mussels) were analyzed for yeast cell fluorescence. Although shrimp and mussel samples from Rainbow site led to the highest rate of isolation, no FISH signal was ever observed. The FISH detection limit of 10^3-10^4 target cells per ml is relatively high (Daims *et al*., 2005) and thus, the absence of FISH signals does not necessarily mean that the target organisms were not present in the samples.

To test this hypothesis, several volumes of water were concentrated on polycarbonate membrane filters to yield sufficient cells for FISH experiments with these new probes. Membrane filters were embedded in low gelling-point agarose to minimize cell loss. Yeast cells could be visualized in a low quantity on these membrane filters (Fig 2). Such results are another evidence of the yeast cells presence in hydrothermal vents but at low concentration. Using FISH on membrane filters, yeast cells detected were affiliated to 3 genera: *Rhodosporidium*, *Rhodotorula* and *Cryptococcus*.

Discussion

Occurrence of yeasts in deep-sea hydrothermal vents

In this study, the main aim was to isolate yeast strains from deep-sea hydrothermal endemic fauna knowing that yeasts can be isolated from seawater surrounding hydrothermal fauna (Gadanho and Sampaio, 2005). Yeast isolation was successful even if the retrieved species richness was relatively low. Thirty-two strains were isolated mostly from *Rimicaris exoculata*

shrimps. The association with shrimps is probably favorable for yeasts that could benefit from nutrients due to the water circulation in the gill chamber. Most of our strains were isolated from the Rainbow hydrothermal site which confirms previous results (Gadanho and Sampaio, 2005). The Rainbow hydrothermal field hosted in ultramafic rocks is a unique vent enriched in CH_4, H_2, CO, Fe and depleted in H_2S (Charlou *et al*., 2002). The high yeast isolation ratio may indicate that yeasts thrive in hydrothermal sites depleted in H_2S. The isolation rate of non-pigmented yeasts on sulfur-free media significantly higher than those on sulfur-based media in a previous study (Gadanho & Sampaio, 2005) support such hypothesis.

Several yeasts were also isolated from mussels and more precisely from the byssus constituted of filaments with a high concentration of minerals and organic matter. These yeasts may have a role in the decomposition of organic material entrapped in mussel byssi in deep-sea vents. These results seem promising as they confirm the data obtained in previous studies and suggest that yeasts may interact with deep-sea hydrothermal vent fauna.

Pattern of the culturable yeast communities

New species.

The yeast that was firstly isolated from stomach of a marine fish was described as *D. hansenii* and deposited in the Centraalbureau voor Schimmelcultures (CBS 5307) database. In a recent paper, based on the intergenic spacer (IGS) region of the rRNA gene, this strain was re-evaluated as *Candida sp*. (Nguyen *et al*., 2009). This strain is identical to another one isolated from deep-sea hydrothermal vent waters and annotated MARY089 (Gadanho and Sampaio, 2005). These two strains isolated from different marine environments were finally reported as a single new undescribed species within the *Candida* genus. In our collection, strain Mo39, isolated from deep-sea coral near Rainbow hydrothermal vents (Table 1), has the same 26S rRNA gene sequence as CBS 5307 and MARY089. Mo39 is halophilic and thus supposed to be an autochtonous marine yeast species. This new ecotype can be characterized as an obligate marine yeast and its complete description is currently under progress.

Known species

Two strains (Mo25 and Mo31) isolated from *Rimicaris exoculata* and *Bathymodiolus azoricus* samples were identified as *Candida atlantica*. This result seems in keeping with previous published reports that have isolated this species from coastal seawater in the South of Portugal (Gadanho *et al*., 2003) and in deep-sea hydrothermal vent waters (Gadanho and Sampaio, 2005). The very fisrt *C. atlantica* strain was isolated from shrimp eggs in the North Atlantic Ocean (Siepmann and Höhnk, 1962). *C. atlantica* seemed to be a marine obligate

yeast and some interactions with shrimps seemed to occur. Our physiological analysis has revealed that Mo25 and Mo31 were non-halophiles, which does not mean that they are unable to grow in marine environments. They may have a role in deep-sea environments in interaction with endemic crustaceans even if they are not in optimal growth conditions. One isolate (Bio1) isolated from seawater surrounding mussels at Lau Basin in the South-West Pacific was clearly identified as *Candida viswanathii*. Kohlmeyer & Kohlmeyer (1979) characterized this yeast as marine facultative. More recently, *C. viswanathii* was isolated from a shrimp (*Peneaus braziliensis*) in the Gulf of Mexico. Its synonym, *Candida lodderae* was recently reported in deep-sea hydrothermal vent waters at Rainbow site (Gadanho and Sampaio, 2005) and characterized as the most abundant yeast.

Leucosporidium scottii isolates (Mo36) were retrieved only in the oceanic regions close to Antarctica and are known to be psychrophilic and probably autochthonous marine species (Lachance and Starmer, 1998). Such strains known for their presence in cold polar marine environments could be another evidence that confirms the hypothesis of global exchanges from polar environments to deep-sea vents based on results from bacteria (Maruyama *et al*., 2000) and filamentous fungi (Burgaud *et al*., 2009). *Hortaea werneckii* (Mo34) was characterized as halophilic in our physiological study. This is not surprising as this black yeast-like fungus was characterized as halophilic or extremely halotolerant in different studies (Gunde-Cimerman *et al*., 2000; Kogej *et al*, 2005) where it was frequently isolated from hypersaline waters of solar salterns. In a molecular survey, *H. werneckii* was identified (based on internal transcribed spacers and 5.8 S rRNA gene) in deep-sea methane seep sediments at a depth of 2965 meters (Lai *et al*., 2007). *Phaeotheca triangularis* (Mo30) was also frequently isolated from salted environments (Gunde-Cimerman *et al*., 2000) and characterized as halophile. This confirmed previous results on *P. triangularis* showing a better growth with 5% additional salts (Zalar *et al*., 1999). In our study, Mo30 was characterized as halotolerant with 4.5% sea salts optimal concentration and thus hypothesized as marine adapted yeast. This is the first report about the presence of *Phaeotheca triangularis* at deep-sea vents.

Mo22 is described as *Sporobolomyces roseus*. The genus *Sporobolomyces* is composed of strains mainly isolated from the pyllophane (Bai *et al*., 2002). However, a previous study has proved that strains of the genus *Sporobolomyces* are frequently isolated from marine ecosystems and the frequency of isolation increases when distance from the coastline and depth increase (Hernandez-Saavedra *et al,* 1992). Moreover, yeasts from this genus were found in benthic invertebrates collected from deep-sea floor in the Pacific Ocean (Nagahama

et al, 2001b). Our strain was isolated from a deep-sea hydrothermal shrimp in the Atlantic Ocean and characterized as halotolerant with an optimal salinity of 6% sea salts. This may indicate that yeasts of this genus are also able to live in deep-sea vents and interact with endemic crustaceans.

A previous study of yeasts in oceanic environments (Fell, 1976) reported that yeast communities appeared to be constituted of ubiquitous and endemic species. Typical ubiquitous strains were the ascomycetous yeast *Debaryomyces hansenii* and the basidiomycetous ones *Cryptococcus* and *Rhodotorula*. Kohlmeyer and Kohlmeyer (1979) confirmed this statement and characterized these genera mainly as facultative marine yeasts. Some of these results, especially for *Rhodotorula* yeasts showing a strong ubiquity, were confirmed based on their presence in several habitats such as deep-sea vents (Gadanho and Sampaio, 2005), deep-sea sediments (Nagahama *et al*., 2001b), coastal waters (Gadanho *et al*., 2003; 2004) and oligotrophic lakes (Libkind *et al*., 2003). Our results confirm their ubiquity and indicate that these strains seem to be allochtonous. Strain Ex15 identified as *Pichia guilliermondii* has also been characterized as non halophile and may be another allochtonous yeast strain as reported by Kohlmeyer and Kohlmeyer (1979).

The members of the genus *Rhodosporidum* have been characterized as non halophiles (Mo24 and Mo33) and halotolerant (Mo38). Based on previous reports, this genus seemed to be restricted to marine environments (Gadanho and Sampaio, 2005). *R. diobovatum* in deep-sea vents seemed to be able to colonize different substrates (shrimps, mussels and sponges). The isolation of a strain from shrimp exuviae in decomposition may indicate a role as a recycler of organic material and so a probable implication in carbon cycle in deep-sea environments.

Adaptation to marine conditions

The isolation of culturable yeasts led to an old question about marine yeasts "Are there any indigenous marine yeasts ?" (Kohlmeyer & Kohlmeyer, 1979) and to the resulting question "Which are the indigenous species ?". Based on our results, one can suggest that halophilic strains are marine indigenous yeasts and that others, halotolerant and non-halophiles, are ubiquitous terrestrial strains present in deep-sea waters due to sedimentation or other natural or anthropogenic phenomena. But almost all yeast species can grow well in media with NaCl concentrations exceeding those normally present in the sea (Kohlmeyer & Kohlmeyer, 1979).

Few yeast species with a physiological dependence on NaCl or other seawater components have been reported (Nagahama, 2006b). Thus, our results appeared in good agreement with such statements. Only 2 strains described as halophiles (Mo34 and Mo39) in our study can be described as obligate marine yeasts.

FISH observations

FISH using labeled oligonucleotide probes targeting rRNA has been used as a powerful technique for assessing both microbial identity and spatial distributions *in situ* in complex environmental contexts (Yang *et al*., 2008). Our results indicate a very low-level of yeasts at deep-sea vents. As a first conclusion, regarding diversity and quantification (added to previous results of Gadanho and Sampaio, 2005), it seems that yeasts at deep-sea vents represent a minor community that might not be major actors in biogeochemical cycles. However, fluorescent signals are correlated to the cellular content of ribosomes and consequently to the microbial growth rates. Recently, the detection limits of conventional FISH with Cy3-labeled probe EUB338 were found to be approximately 370 16S rRNA molecules per cell for *Escherichia coli* hybridized on glass microscope slides and 1,400 16S rRNA copies per *E. coli* cell in environmental samples (Hoshino *et al*., 2008). So, in addition to a low concentration of yeast cells, low detection of yeasts may be caused by low ribosome content of most yeasts in the deep-sea environment due to low-level metabolic activities of yeasts living under extreme environmental abiotic factors (high hydrostatic pressure, low temperatures,…). Our attempts to cultivate the yeast strains resulted from this study under elevated hydrostatic pressure have been successful, but ribosomal activities were lower under high hydrostatic pressure than at atmospheric pressure. Such results may account for the low fungal detection using FISH (unpublished data). Consequently, care must be taken when dealing with diversity and biomass estimations when using FISH alone.

The quantification of yeasts using FISH has been impossible due to a non homogeneous repartition of microorganisms on filters. Moreover, bacterial and yeast cells were only visible in some regions of the filters without minerals due to strong autofluorescence. However we can say that yeast concentrations are really low, as shown by the only few cells visualized after filtration of seawater surrounding shrimps. This result is in keeping with the relatively low diversity revealed by Gadanho and Sampaio (2005) ranging from 0 to 10 cfu/L for pink yeasts and from 0 to 6000 cfu/L for non-pigmented yeasts. To better analyze the fungal presence in deep-sea animals, one could work with phylum-specific probes on histological

sections of animals and use the CARD-FISH (Amann & Fuchs, 2008) or the DOPE-FISH (Stoecker *et al*., 2010) methods to amplify probe signals.

These data raise emerging questions regarding the ecological role of such microorganisms in deep-sea vents and about the old question of the ubiquity or endemism of those strains. Yeasts at deep-sea vents may be facultative parasites or opportunistic pathogens of endemic deep-sea animals as it has already been hypothesized in previous works (Van Dover *et al*., 2007; Burgaud *et al*, 2009). However, a role in th edecomposition of abundant organic material may occur.

Considering all the results obtained, we can say that yeasts may seem to interact with deep-sea hydrothermal endemic fauna even if the density is low. These yeasts are mainly composed of ubiquitous species but obligate marine yeasts have also been harvested. However, the results obtained using *in situ* hybridization have allowed us to visualize these ubiquist species showing that they are able to live and grow in deep-sea hydrothermal vents. Yeasts associated with endemic animals in deep-sea vents may be exposed to favorable conditions and could benefit from a stable source of nutrients (Nagahama *et al*., 2001b). Yeasts were reported from dead and healthy individuals which may also indicate their facultative saprophytism and so emphasize the wide role of fungi in the decomposition of organic matter from terrestrial environments to deep-sea hydrothermal vents. Even if yeasts were isolated from animal body components, they were not visualized using FISH. To better understand the interaction with animals and fungi in deep-sea vents, we need to work on tissues as in Van Dover *et al*. (2007) and also with probes specific to fungal phyla (*Ascomycota*, *Basidiomycota* and *Chytridiomycota*). In conclusion, several questions regarding the role of yeasts in deep-sea hydrothermal vents and the endemism or ubiquity of the isolated yeasts remain a difficult task without clear answers. Their culture under high hydrostatic pressures would be an interesting study to better characterize their lifestyle and role at deep-sea vents.

Acknowledgements

We thank the chief scientists of the BIOLAU, DIVANAUT2, HERO, MARVEL, EXOMAR and MoMARDREAM-Naut cruises, the pilots and support crews of the oceanographic vessels and Deep Submergence Vehicles of Ifremer. We greatly acknowledge Dr Jerome Mounier for his valuable help and comments on FISH and Jerome Lepioufle for valuable advice on the manuscript. We thank all the members of the GDR Ecchis for their advice and suggestions. We would like to thank the editor and the anonymous reviewers who have provided helpful

559 comments on the refinement of this manuscript. We also thank ANR Deep-Oases, Ifremer,
560 Region Bretagne and French Research Ministry for their financial support. We finally thank
561 Françoise Gaill and the CHEMECO/European Science Foundation EURODEEP for
562 discussions and financial support.
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Tables and Figures

Table 1. Culture collection of yeasts from deep-sea hydrothermal vents.

Location (Depth)	Sample processed (type)	Strain
South Pacific West;	B2E07: Seawater surrounding mussels	Bio1
(*Lau Basin*; -2620m)	B9E07: Gastropod (*Ifremeria nautilei*) gills	Bio2
Mid-Atlantic Ridge	EX6E01 to EX6E04: *Rimicaris exoculata*	Ex2 to Ex7
(*Rainbow*; -2300m)	EX6E05: *Chorocaris chacei*	Ex9, Ex11 and Ex12
	MoPR1: *Rimicaris exoculata*	Mo20
	MoPR1: *Mirocaris fortunata*	Mo21
	MoPR2: *Rimicaris exoculata*	Mo22
	MoPR3: Sloughs of shrimp on smocker rocks	Mo24 and Mo25
	MoPR5: Colonization module TRAC (Carbonates)	Mo26 to Mo29
	MoPR6: *Bathymodiolus azoricus*	Mo30 to Mo36
	MoPR8: *Rimicaris exoculata*	Mo37
	MoPR9: Sponge	Mo38 and Mo39
	MoPR9: Coral	Mo40
Mid-Atlantic Ridge	EX18E02: Siliceous sponge	Ex15
(*Lost-City*; -700m)		

796 Table 2. Physiological analysis of the yeast collection. This table shows distribution of
797 halotolerant and halophilic strains of the collection depending on their optimal salinities (g/l
798 sea salts), optimal temperatures (°C) and maximal optical densities of cultures on GYPS broth
799 medium (120 rpm on a rotary shaker) measured at 600nm at 4 different incubation times (17h,
800 22h, 25h and 28h).

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		Low OD (<1.1)	High OD (>2.0)	
	Optimum	**25°C**	**25°C**	**35°C**
Non halophile	**0-15 g/l**	Mo25	Bio1, Bio2, Ex2, Ex3, Ex4, Ex5, Ex6, Ex7, Ex9, Ex11, Ex12, Mo20, Mo21, Mo24, Mo26, Mo27, Mo28, Mo29, Mo31, Mo32, Mo33, Mo35, Mo40	Ex15
Halotolerant	**30 g/l**	Mo36	Mo37, Mo38	
	45 g/l			Mo30
	60 g/l	Mo22		
Halophile	**30 g/l**	Mo34		Mo39

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806 Table 3. Yeast oligonucleotide probes and their sequences, target organisms and binding
807 positions on the 26S rRNA.

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Probe	**Hybridization stringency (% formamide)**	**rRNA subunit, binding position [(a)] and relative probe accessibility [(b)]**	**Probe sequence (5'-3')**	**Target organisms (Genus/Species)**
Sacch	20	26S; 162-177 ; 44 to 66%	GGCATCTCATCGCACG	*Debaryomyces* *Pichia*
MitoFilo	10	26S; 397-412 ; 60%	ACACCGCAGAACGGCT	Members of the genus *Cryptococcus* [(c)]
MitoSporidio	20	26S; 164-179 ; 44 to 66%	TGGGCGTCCGCACCAT	Members of the genera *Rhodotorula* and *Rhodosporidium* [(d)]

(a) Nucleotide position according to *Saccharomyces cerevisiae* 26Sr RNA between NL1 and NL4 primers.

(b) According to Inacio *et al*., 2003.

(c) *Cryptococcus saitoi*, *C. randhawii*, *C. uzbekistanensis*, *C. adeliensis*, *C. vishniacii*, *C. socialis*, *C. friedmannii* and *C. uniguttulatus*.

(d) *Rhodotorula mucilaginosa*, *R. glutinis*, *R. graminis*, *R. dairenensis*, *Rhodosporidium babjevae* and *R. diobovatum*.

Figure 1: Phylogenetic tree of deep-sea yeast isolates (coloured terminals) and close relatives obtained by analysis of the D1/D2 domain of the 26S rRNA gene. Topology was built using MrBayes v.3.1.2 from a ClustalW 1.83 alignment. Node support values are given in the following order: MrBayes posterior probabilities/PHYML 100 bootstraps. Black squares represent nodes supported by an excess of 0.95 posterior probabilities and 95% bootstraps. *Mucor flavus* (EU071390) belonging to the *Zygomycota* phylum was used as outgroup. All sequences are listed with their GenBank accession numbers.

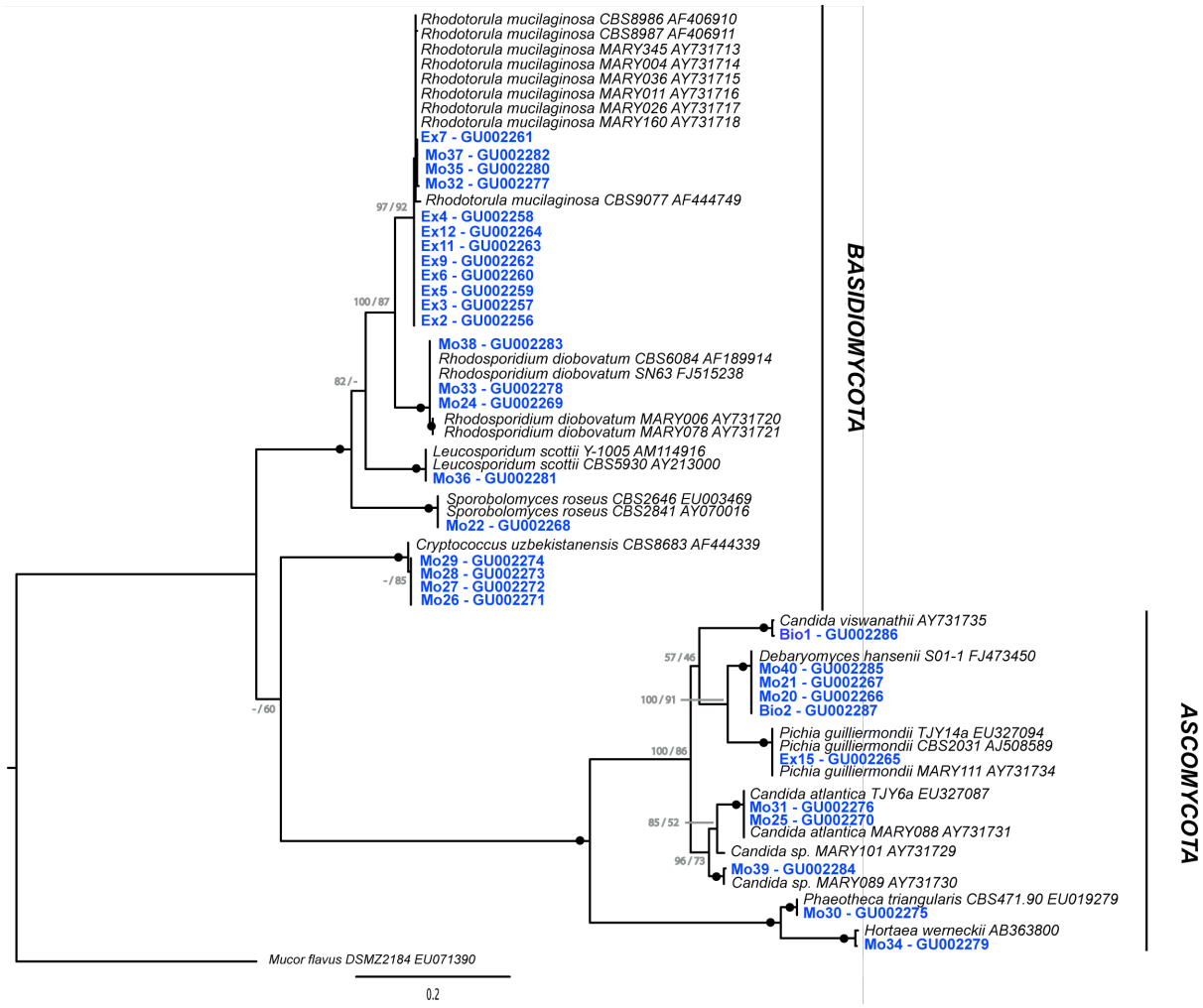

Rhodotorula mucilaginosa CBS8986 AF406910
Rhodotorula mucilaginosa CBS8987 AF406911
Rhodotorula mucilaginosa MARY345 AY731713
Rhodotorula mucilaginosa MARY004 AY731714
Rhodotorula mucilaginosa MARY036 AY731715
Rhodotorula mucilaginosa MARY011 AY731716
Rhodotorula mucilaginosa MARY026 AY731717
Rhodotorula mucilaginosa MARY160 AY731718
Ex7 - GU002261
Mo37 - GU002282
Mo35 - GU002280
Mo32 - GU002277
Rhodotorula mucilaginosa CBS9077 AF444749
97 / 92
Ex4 - GU002258
Ex12 - GU002264
Ex11 - GU002263
Ex9 - GU002262
Ex6 - GU002260
Ex5 - GU002259
Ex3 - GU002257
Ex2 - GU002256
100 / 87
Mo38 - GU002283
Rhodosporidium diobovatum CBS6084 AF189914
Rhodosporidium diobovatum SN63 FJ515238
82 / -
Mo33 - GU002278
Mo24 - GU002269
Rhodosporidium diobovatum MARY006 AY731720
Rhodosporidium diobovatum MARY078 AY731721
Leucosporidium scottii Y-1005 AM114916
Leucosporidium scottii CBS5930 AY213000
Mo36 - GU002281
Sporobolomyces roseus CBS2646 EU003469
Sporobolomyces roseus CBS2841 AY070016
Mo22 - GU002268
Cryptococcus uzbekistanensis CBS8683 AF444339
Mo29 - GU002274
Mo28 - GU002273
- / 85
Mo27 - GU002272
Mo26 - GU002271
BASIDIOMYCOTA
Candida viswanathii AY731735
Bio1 - GU002286
57 / 46
Debaryomyces hansenii S01-1 FJ473450
Mo40 - GU002285
Mo21 - GU002267
100 / 91
Mo20 - GU002266
Bio2 - GU002287
- / 60
Pichia guilliermondii TJY14a EU327094
Pichia guilliermondii CBS2031 AJ508589
100 / 86
Ex15 - GU002265
Pichia guilliermondii MARY111 AY731734
Candida atlantica TJY6a EU327087
Mo31 - GU002276
85 / 52
Mo25 - GU002270
Candida atlantica MARY088 AY731731
Candida sp. MARY101 AY731729
96 / 73
Mo39 - GU002284
Candida sp. MARY089 AY731730
Phaeotheca triangularis CBS471.90 EU019279
Mo30 - GU002275
Hortaea werneckii AB363800
Mo34 - GU002279
ASCOMYCOTA
Mucor flavus DSMZ2184 EU071390
0.2

Figure 2: Fluorescence *in situ* hybridization with specific oligonucleotide probes on membrane filters. (a, b and c) Membrane filter labelled with DAPI and hybridized using MitoSporidio probe indicating the presence of bacteria and yeast cells (blue). Yeasts belonging to *Rhodotorula* and *Rhodosporidium* genera are vizualized in pink (composite of blue and red). (d) Membrane filter labelled with DAPI and hybridized with MitoFilo indicating the presence of yeasts belonging to *Cryptococcus* genera. White arrows indicate the yeast cells.

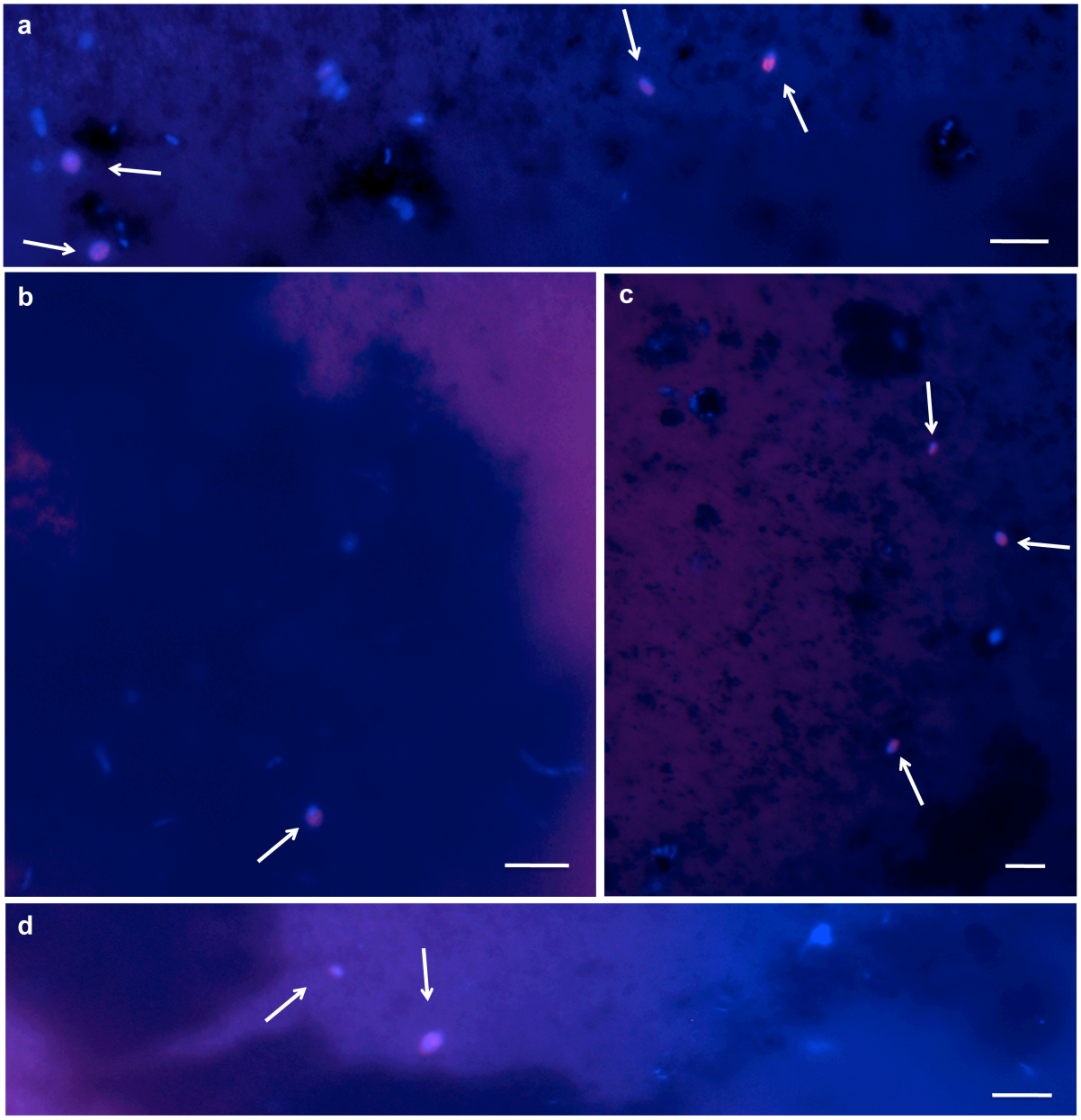
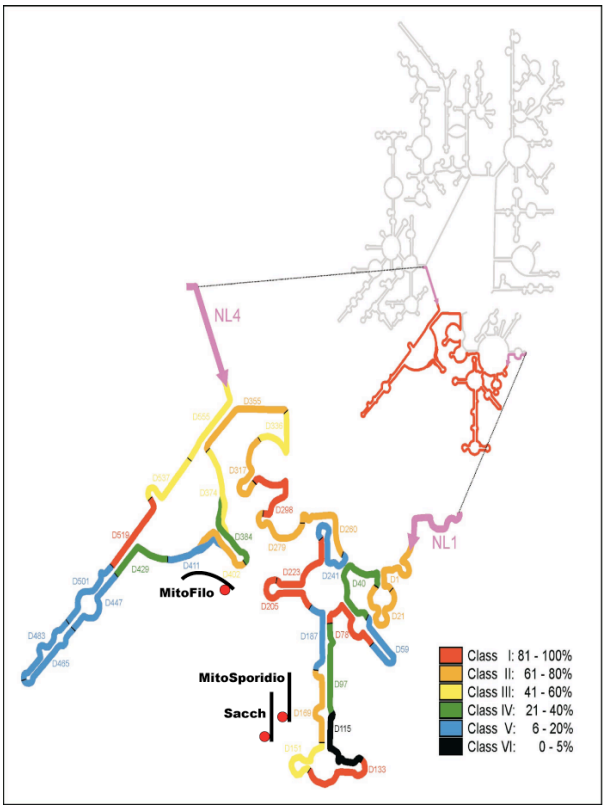
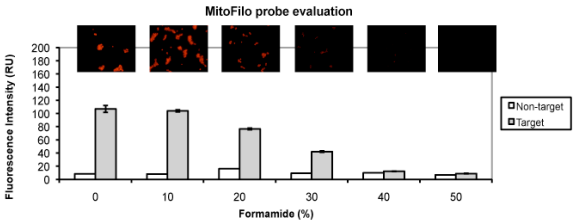
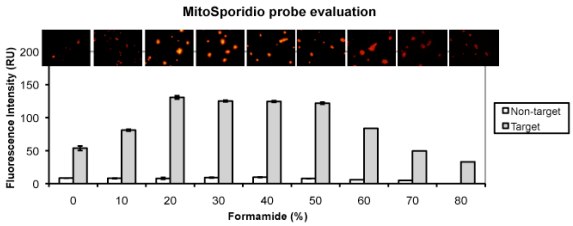
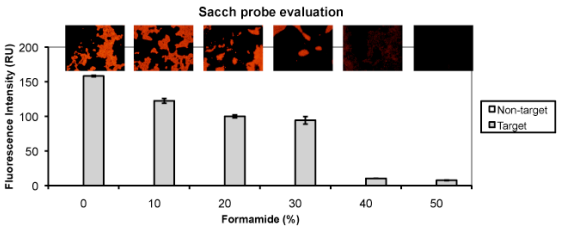

a
b
c
d

Figure S1 : Target sites of the fluorescent oligonucleotide probes designed on a model of the *Saccharomyces cerevisiae* 26S rRNA secondary structure in which the D1 and D2 domains (delimited by NL1 and NL4) are enlarged (Inacio *et al*., 2003). Each probe was evaluated without formamide in order to check wether the probe binds to the ribosomes of the target cells. The optimal hybridization conditions were determined in a serie of FISH experiments with increasing formamide concentrations for a probe target and a non-target organism : (i) Sacch probe, *Debaryomyces hansenii* (Target) and *Candida atlantica* (Non-Target) with two mismatches ; (ii) MitoSporidio probe, *Rhodosporidium diobovatum* (Target) and *Cryptococcus uzbekistanensis* (Non-Target) with five mismatches and (iii) MitoFilo probe, *Cryptococcus uzbekistanensis* (Target) and *Rhodosporidium diobovatum* (Non-Target) with seven mismatches. Relative probe accessibility was determined for each probe : MitoFilo, about 60%; MitoSporidio and Sacch, 44 to 66%.


NL4
NL1
MitoFilo
MitoSporidio
Sacch
Class I: 81 - 100%
Class II: 61 - 80%
Class III: 41 - 60%
Class IV: 21 - 40%
Class V: 6 - 20%
Class VI: 0 - 5%


MitoFilo probe evaluation
Fluorescence Intensity (RU)
Formamide (%)
Non-target
Target


MitoSporidio probe evaluation
Fluorescence Intensity (RU)
Formamide (%)
Non-target
Target


Sacch probe evaluation
Fluorescence Intensity (RU)
Formamide (%)
Non-target
Target