RESEARCH ARTICLE

Marine culturable yeasts in deep-sea hydrothermal vents: species richness and association with fauna

Gaëtan Burgaud[1], Danielle Arzur[1], Lucile Durand[2], Marie-Anne Cambon-Bonavita[2] & Georges Barbier[1]

[1]Laboratoire Universitaire de Biodiversité et Ecologie Microbienne (EA3882), IFR 148, Université Européenne de Bretagne, Université de Brest, ESMISAB, Technopole Brest-Iroise, Plouzané, France; and [2]Ifremer, Centre de Brest, DEEP/LM2E, UMR 6197, Plouzané, France

Correspondence: Gaëtan Burgaud, Laboratoire Universitaire de Biodiversité et Ecologie Microbienne (EA3882), IFR 148, Université Européenne de Bretagne, Université de Brest, ESMISAB, Technopole Brest-Iroise, 29280 Plouzané, France. Tel.: +33 2 98 05 61 48; fax: +33 2 98 05 61 01; e-mail: gaetan.burgaud@univ-brest.fr

Received 9 October 2009; revised 16 March 2010; accepted 16 March 2010.
Final version published online 4 May 2010.

DOI:10.1111/j.1574-6941.2010.00881.x

Editor: Michael Wagner

Keywords
yeasts; hydrothermal vents; fauna; 26S rRNA gene; FISH.

Abstract

Investigations of the diversity of culturable yeasts at deep-sea hydrothermal sites have suggested possible interactions with endemic fauna. Samples were collected during various oceanographic cruises at the Mid-Atlantic Ridge, South Pacific Basins and East Pacific Rise. Cultures of 32 isolates, mostly associated with animals, were collected. 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 the ocean surface to the deep sea. Marine yeasts are divided into *obligate* and *facultative* groups. Obligate marine yeasts are yeasts that have never been isolated from anywhere other than the marine environment, whereas facultative marine yeasts are also known from terrestrial habitats (Kohlmeyer & Kohlmeyer, 1979). Based on these definitions, Kohlmeyer & Kohlmeyer (1979) examined yeasts occurring in marine environments and gathered a list of 176 species isolated from diverse marine habitats. Of those, only 25 were obligate marine yeasts, widely represented by the genera *Metschnikowia*, *Rhodosporidium*, *Candida* and *Torulopsis*.

Hawksworth (2002) hypothesized the existence of 1.5 million fungal species; this estimate is now a commonly used and accepted figure. If this is correct, $< 5\%$ of the fungi have been described up to now and these almost exclusively from terrestrial environments. In that 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 these 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 seafloor 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 of 6–23 °C into seawater at 2–4 °C when hot vents called black smokers 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

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grow and interact with all biotic or abiotic components of these ecosystems. Dense animal communities cluster around these hot springs. These communities are supported by the chemolithoautotrophic activities of prokaryotes (Joergensen & Boetius, 2007).

The occurrence of fungi (filamentous fungi and yeasts) at deep-sea hydrothermal vents remains an underexplored topic. Over the last few years, interest in the diversity of microbial eukaryotes in these ecosystems has increased, using PCR amplification of SSU rRNA 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 dealt specifically 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 have also been published. Bass *et al.* (2007) reported the presence of sequences affiliated to *Debaryomyces hansenii* and novel sequences similar to *Malassezia furfur* in hydrothermal sediments. Le Calvez *et al.* (2009) reported that fungal diversity from deep-sea vent animals was widely composed of sequences affiliated to *Chytridiomycota* and *Basidiomycota* phyla. The latter phylum was mostly represented by yeasts of, for example, the *Cryptococcus* and *Filobasidium* genera, which form dense clusters.

The occurrence of yeasts in other deep-sea environments has been studied to a much greater extent. Nagahama *et al.* (2001b) reported that culturable fungal diversity was dominated by ascomycetous yeasts in surface sediments in water depths exceeding 2000 m (*Candida*, *Debaryomyces*, *Kluyveromyces*, *Saccharomyces* and *Williopsis*). Inversely, diversity was dominated by basidiomycetous yeasts on the subsurface of sediments in water depths exceeding 2000 m and by 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 suggested 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, b, 2006, 2008).

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

Materials and methods

Environmental sampling

We collected 210 hydrothermal samples during six oceanographic cruises at several dates and locations (for hydrothermal vents locations, see Tivey, 2007): (1) BIOLAU in the Lau Basin, South-west Pacific (12/05/1989–27/05/1989; 20°3.0′S, 176°7.8′W; −2620 m); (2) 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; (3) HERO on the EPR at Elsa site (30/09/1991–04/11/1991; 12°48′N, 103°57′W; −2630 m); (4) MARVEL (29/08/1997–13/09/1997) on the MAR at Menez Gwen and Lucky Strike sites; (5) 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; (6) 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 vessels.

The deep-sea samples were processed as described by Burgaud *et al.* (2009) taking care to avoid contamination by applying strict sterile sampling conditions.

Enrichment conditions and isolation

The samples were processed directly after the *Nautile* or ROV recovery. The collected samples were mainly composed of deep-sea hydrothermal vent animals (*Rimicaris exoculata* and *Chorocaris chacei* shrimps and *Bathymodiolus azoricus* mussels) and were used to inoculate the GYPS culture medium which had led to the best isolation rate during a previous study (Burgaud *et al.*, 2009). This medium contained per liter: glucose (Sigma) 1 g, yeast extract (AES) 1 g, peptone (AES) 1 g, starch (Fisher) 1 g, sea salts (Sigma) 30 g. This medium was supplemented per liter with agar 15 g and chloramphenicol (Sigma) 500 mg; pH was also adjusted to 7.5. Cultures were done aerobically at 4, 15 and 25 °C (ambient temperature), and at 35 and 45 °C (only during EXOMAR) at atmospheric pressure until fungal strains were visualized. During the MoMARDREAM-Naut cruise, some dissections were done on board on animal samples in order to investigate the yeast location.

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

Locations (depth)	Sample processed (type)	Strains
South Pacific West (Lau Basin, −2620 m)	B2E07: seawater surrounding mussels	Bio1
	B9E07: gastropod (*Ifremeria nautilei*) gills	Bio2
Mid-Atlantic Ridge (Rainbow, −2300 m)	EX6E01 to EX6E04: *Rimicaris exoculata*	Ex2–Ex7
	EX6E05: *Chorocaris chacei*	Ex9, Ex11 and Ex12
	MoPR1: *Rimicaris exoculata*	Mo20
	MoPR1: *Mirocaris fortunata*	Mo21
	MoPR2: *Rimicaris exoculata*	Mo22
	MoPR3: sloughs of shrimp on smoker rocks	Mo24 and Mo25
	MoPR5: colonization module TRAC (carbonates)	Mo26 –Mo29
	MoPR6: *Bathymodiolus azoricus*	Mo30–Mo36
	MoPR8: *Rimicaris exoculata*	Mo37
	MoPR9: sponge	Mo38 and Mo39
	MoPR9: coral	Mo40
Mid-Atlantic Ridge (Lost City, −700 m)	EX18E02: siliceous sponge	Ex15

All purified strains from our collection (Table 1) have been integrated to the Souchothèque de Bretagne culture collection (http://www.ifremer.fr/souchotheque/internet/htdocs/generique.php?pagebody=catalogue.php) and are available with an accession number associated to their GenBank number.

Physiological characterization and statistical analysis

All experiments were done in triplicate. The yeasts were grown in liquid GYPS broth media. The effect of temperature on growth was determined at 5, 15, 25 and 35 °C at 30 g L^{-1} sea salts. The effect of salinity was analyzed modifying sea salt concentrations in media from 0 to 60 g L^{-1} with steps of 15 g L^{-1} at the optimal temperature for each strain. ODs were measured at 600 nm with Nanocolor 100D (Macherey-Nagel, Hoerdt, France) at 17, 22, 25 and 28 h of growth for salinity and temperature levels.

DNA extraction and 26S rRNA gene sequencing

DNA of each strain was extracted using FastDNA Spin Kit (MP Biomedicals, Illkirch, France) specific for fungi and yeasts. Amplifications of the D1/D2 region of 26S rRNA gene were carried out with rRNA gene primers ITS5 (5′-GGA AGT AAA AGT CGT AAC AAG-3′), LR6 (5′-CGC CAG TTC TGC TTA CC-3′), NL1 (5′-GCA TAT CAA TAA GCG GAG GAA AAG-3′) and NL4 (5′-GGT CCG TGT TTC AAG ACG G-3′) as described by Gadanho & Sampaio (2005). All PCR reactions were performed in 20-μL reaction volumes containing 19 μL of 1 × PCR buffer (Promega), 2 mM of $MgCl_2$, 0.2 mM of each dNTP (Promega), 0.6 μM of primers (forward and reverse), 1.25 U of Taq DNA Polymerase (Promega) and 1 μL of DNA. The PCRs were performed on PTC-200 (BioRad, France). The amplification consisted of an initial denaturation step at 94 °C for 2 min, followed by 30 iterations of 15 s at 94 °C, 30 s at 54 °C, 1 min at 72 °C and a final extension step of 2 min at 72 °C. A negative control with sterile-distilled water replacing DNA was added. Two kinds of amplification were generated using ITS5-NL4 and NL1-LR6 primers. The amplified DNA fragments were separated by electrophoresis in 0.8% agarose (w/v) gel (Promega) in 0.5 × Tris–borate–EDTA buffer at 90 V for 1 h and stained with ethidium bromide. A molecular size marker was used for reference (Lambda DNA/EcoR1+Hind III Markers, Promega). The DNA banding patterns were visualized under UV transillumination and picture files were generated using GEL-DOC 2000 (BioRad).

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

Phylogenetic analyses

Sequences were edited and cleaned using SEQUENCHER v. 4.8 (Gene Codes). Sequences were then imported to MEGA 4.0 software (Tamura *et al.*, 2007). Each sequence was analyzed to find GenBank sequences with close BLAST-N hits (Altschul *et al.*, 1990). Similarities between sequences were assessed using pairwise distance calculation with MEGA 4.0. The sequences were trimmed to ensure that all sequences had the same start and end point. All the D1/D2 regions of the 26S rRNA gene sequences were aligned using CLUSTALW v. 1.83 (Thompson *et al.*, 1994). After visual checking and manual curation, an alignment composed of 62 taxa and 579 characters was analyzed for the Bayesian estimation of phylogeny using MRBAYES v. 3.1.2 software (Ronquist & Huelsenbeck, 2003). A 2-million generation option has been set to run the Metropolis-coupled Monte Carlo Markov

chain (*mcmc*) method. After generation 2 000 000, the SD of split frequencies was $P = 0.005997$, indicating that a convergence had occurred (P-value of < 0.05). The alignment was analyzed using MODELTEST v. 3.7 (Posada & Crandall, 1998) to obtain the more realistic evolutionary model used for phylogenetic analyses (GTR+G model; gamma-distribution shape parameter = 0.3978). Phylogeny was then evaluated using two different methods:

(1) Bayesian inference with MRBAYES v. 3.1.2 analysis was done using 2 000 000 generations and the *mcmc* method. The tree search included two *mcmc* searches with four chains (setting a default temperature for heating the chains) and a sampling frequency of 100 generations. A 'burnin' of 5000 (25% of the 2 000 000 generations/100 sample frequency) was set to exclude the first 5000 trees generated.

(2) Maximum likelihood (ML) with 100 bootstrap iterations was carried out 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 PRIMROSE software (http://www.bioinformatics-toolkit.org/Primrose/index.html) as described by Ashelford *et al.* (2002) with a set of high-quality, full-length rRNA gene sequences of probe target organisms. The PRIMROSE design tool produced oligonucleotide probes for the three principal clusters of our collection (Table 2). These probes exhibited no mismatches with the target organisms but did exhibit mismatches with the next most similar sequences in the GenBank database, proving that the designed probes were highly specific *in silico*. The target sites of newly designed probes were checked for accessibility using the prediction maps based on the 26S rRNA gene of *Saccharomyces cerevisiae* (Inacio *et al.*, 2003). Each probe was in a relatively accessible area of the 26S rRNA gene secondary structure (Supporting Information, Fig. S1). As it was not possible to test the probes with culture isolates that exhibited no 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 oligonucleotides. 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 $-20\,^{\circ}$C. The newly designed probes were checked under *in situ* conditions with target and nontarget species. The universal probe Euk516-Fluorescein (5′-ACCA GACTTGCCCTCC-3′; Amann *et al.*, 1995) and the non-Euk516-Cy3 (5′-CCTCCCGTTCAGACCA-3′) probes were used as positive and negative controls, 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 DAIME software (Daims *et al.*, 2006). During this step, the intensities of the image pixels analyzed, enabled determination of single cell fluorescence in relative units.

FISH

On environmental samples

Interior branchiostegites of *R. exoculata* shrimps and byssus of *B. 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 h at 4 °C in a dark room. After fixation, tissues were washed three times with PBS and

Table 2. Yeast oligonucleotide probes and their sequences, target organisms and binding positions on the 26S rRNA gene

Probe	Hybridization stringency (% formamide)	rRNA subunit, binding position* and relative probe accessibility†	Probe sequence (5′–3′)	Target organisms (genus/species)
Sacch	20	26S; 162–177; 44–66%	GGCATCTCATCGCACG	*Debaryomyces*, *Pichia*
MitoFilo	10	26S; 397–412; 60%	ACACCGCAGAACGGCT	Members of the genus *Cryptococcus*‡
MitoSporidio	20	26S; 164–179; 44–66%	TGGGCGTCCGCACCAT	Members of the genera *Rhodotorula* and *Rhodosporidium*§

*Nucleotide position according to *Saccharomyces cerevisiae* 26S rRNA gene between NL1 and NL4 primers.

†According to Inacio *et al.* (2003).

‡*Cryptococcus saitoi*, *Cryptococcus randhawii*, *Cryptococcus uzbekistanensis*, *Cryptococcus adeliensis*, *Cryptococcus vishniacii*, *Cryptococcus socialis*, *Cryptococcus friedmannii* and *Cryptococcus uniguttulatus*.

§*Rhodotorula mucilaginosa*, *Rhodotorula glutinis*, *Rhodotorula graminis*, *Rhodotorula dairenensis*, *Rhodosporidium babjevae* and *Rhodosporidium diobovatum*.

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) for 2 h at 4 °C. Fixed seawater was then filtered on 0.22-μm polycarbonate membranes (Nuclepore®, 47 mm diameter; Whatman, Maidstone, UK) and rinsed with a PBS 2 × /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 pasted onto slides with one drop of 0.2% low-gelling point agarose (35–40 °C) (Menzel-Glaser, Germany). All slides were then dipped in 0.2% agarose and air dried. Samples were then subjected to dehydration with increasing concentrations of ethanol (50%, 80% and 96%, for 3 min each). The concentration of working solutions of probes was 30 ng DNA L^{-1}. 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 probes were gently mixed in a ratio of 10 : 1 (v/v) to obtain a final oligonucleotide concentration of 3 ng L^{-1}. For hybridization, slides were placed in sampling tubes and incubated at 46 °C in the dark for exactly 3 h. 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^{-1}) and mounted with the antifading reagent Citifluor AF 2 (Citifluor, France) before observation under a fluorescent microscope.

Results

Yeast isolation

Yeasts were not found in all the studied sites (Table 1). No yeast was isolated from samples collected during HERO (on the East Pacific Rise at Elsa site), DIVANAUT2 and MARVEL (Menez Gwen 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 from 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 32 isolates that could be divided in pigmented yeasts ($n = 18$) and nonpigmented yeasts ($n = 14$). Pigmented yeasts consisted mainly of red-pigmented yeasts ($n = 16$) with one black-pigmented yeast and one brown-pigmented yeast.

Regarding yeast isolation vs. type of substrate, strains were obtained mostly from hydrothermal shrimps *R. exoculata* ($n = 11$), *C. chacei* ($n = 3$) and *Mirocaris fortunata* ($n = 1$), and from hydrothermal mussels *B. azoricus* ($n = 7$). Carbonate colonization modules deployed for 1 year near Rainbow vent yielded a few yeasts ($n = 4$); and three yeasts were isolated from sponges. One strain each was obtained from seawater, gastropod and coral samples (Table 1). These results indicate that the yeasts were associated much more with animals than with mineral substrates. Statistical distribution tests were performed to determine 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 from 1 correspond to $(s2/m) - 1 > 2[2n/(n-1)2]1/2$ and were obtained only for the 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, which can be divided into three different compartments: (1) an antero-ventral area, which was relatively clear; (2) a posterior area, which always remained light beige; and (3) an antero-dorsal area, with an intensely rusty coloration (for schematic views, see Zbinden *et al.*, 2004; Corbari *et al.*, 2008). Yeast isolates from this study were all cultivated from the antero-dorsal area, which was characterized by high amounts of minerals and a dense bacterial mat.

Seven yeasts were also isolated from *B. azoricus* during the MoMADREAM-Naut oceanographic cruise (Table 1). Six of them were cultivated from the external face of the mussel shells, more precisely from the byssus, which is a network of filaments that allows attachment to rocks. This tangle gathers many particles and organic matter in decomposition (G. Burgaud, personal observation). Only one yeast was isolated from the interior of a mussel (Mo32).

Physiological analysis

Three categories of strains were identified (Table 3) based on the definition of halotolerant and halophilic microorganisms (Kushner, 1978; Margesin & Schinner, 2001). Nonhalophiles are strains with maximal growth without sea salts and a decreasing growth rate with increasing concentrations of sea salts in media. Halotolerant yeasts are strains able to grow in the absence as well as in the presence of salt. Halophiles require salt for an optimal growth. Regarding halophily, optimal salinities, optimal temperatures and OD measurement, nine physiological groups were defined. Most of the isolated strains were nonhalophiles (23 strains) and halotolerant (two strains, maximal OD at 30 g L^{-1} sea salts) growing efficiently at an optimal temperature of 25 °C. Four strains had poor maximal growth at 25 °C, including one nonhalophile, two halotolerant (maximal OD at 30 and 60 g L^{-1} sea salt) and one halophile (maximal OD at 30 g L^{-1} sea salt). Three strains had maximal and efficient growth at 35 °C, including one nonhalophile, one halotolerant (maximal OD at 45 g L^{-1} sea salts) and one halophile (maximal OD at 30 g L^{-1} sea salts).

Identification

A sequence analysis of the D1/D2 domain of the 26S rRNA gene was done to identify species (Fig. 1). Twelve phylotypes were 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, comprising 16 strains. The majority of strains (Ex2, Ex3, Ex4, Ex5, Ex6, Ex7, Ex9, Ex11, Ex12, Mo32, Mo35 and Mo37) were identified as *Rhodotorula mucilaginosa* (100% similarity). Most of the *R. mucilaginosa* were isolated from deep-sea shrimps ($n = 14$) and the others from deep-sea mussels ($n = 2$). As a member of the *Sporidiobolales* order, isolates affiliated to *Rhodosporidium diobovatum* were also isolated (Mo24, Mo33 and Mo38) with 100% similarity. These three strains were isolated, respectively, from *R. exoculata* exuviae in decomposition on smoker rocks, *B. 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 contained nine strains belonging to the *Saccharomycetales* order. Within this order, four strains (Mo20, Mo21, Mo40 and Bio2) isolated, respectively, from *R. exoculata*, *M. fortunata*, a deep-sea coral and the gills of the gastropod *Ifremeria nautilei* were identified as *D. 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 *B. azoricus* was identified as *Phaeotheca triangularis* (mitosporic *Ascomycota*) with 100% similarity. In the *Dothideales* order, one strain (Mo34) isolated from *B. azoricus* was identified as *Hortaea werneckii* with 99.98% similarity (one mismatch on 560 bp).

Sequencing of the 26S rRNA genes indicated the presence of *Ascomycota* and *Basidiomycota* in our culture collection. There were twice as many of the *Basidiomycota* phylum ($n = 21$) as of the *Ascomycota* phylum ($n = 11$). In terms of species richness, ascomycetous yeasts belonged to seven different clusters, whereas basidiomycetous yeasts belonged to five.

Table 3. Physiological analysis of the yeast collection

		Low OD (< 1.1)	High OD (> 2.0)	
	Optimum (g L^{-1})	25 °C	25 °C	35 °C
Nonhalophilic	0–15	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	Mo36	Mo37, Mo38	
	45			Mo30
	60	Mo22		
Halophilic	30	Mo34		Mo39

This table shows distribution of halotolerant and halophilic strains of the collection depending on their optimal salinities (g L^{-1} sea salt), optimal temperatures (°C) and maximal ODs of cultures on GYPS broth medium (120 r.p.m. on a rotary shaker) measured at 600 nm at four different incubation times (17, 22, 25 and 28 h).

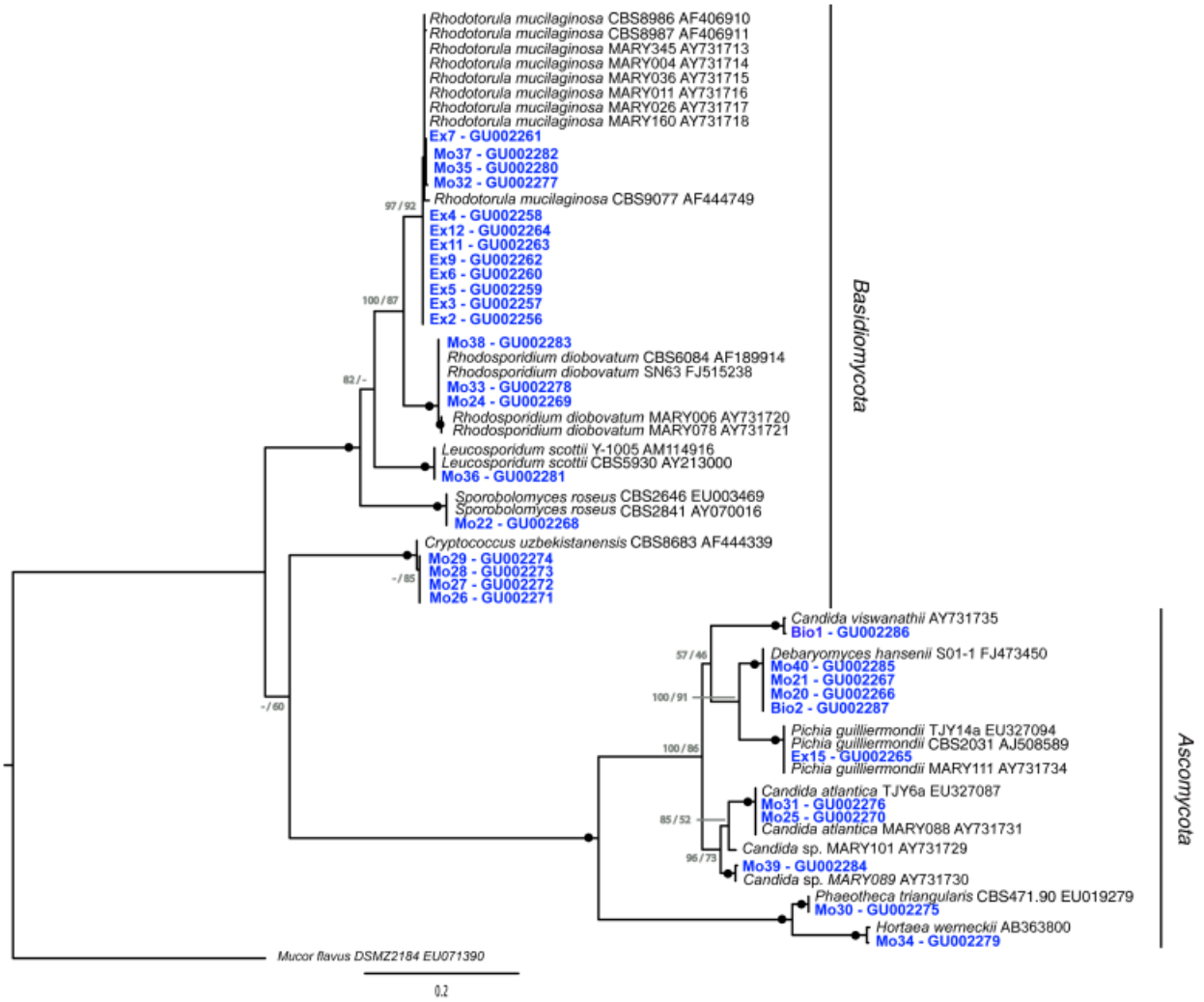

Fig. 1. Phylogenetic tree of deep-sea yeast isolates and close relatives obtained by analysis of the D1/D2 domain of the 26S rRNA gene. Topology was built using MRBAYES v. 3.1.2 from a CLUSTALW 1.83 alignment. Node support values are given in the following order: MRBAYES posterior probabilities/PHYML 100 bootstraps. Black circles 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.

FISH

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, and so was not used. The probe MY1574 targeting *Eumycota* organisms (Baschien *et al*., 2008) showed very weak fluorescence on pure cultures. We therefore decided to design our own probes (Table 2) based on our culture collection, divided into three main clusters: MitoFilo (*Cryptococcus*/Mitosporic *Filobasidiales* order), MitoSporidio (*Rhodotorula*, *Rhodosporidium*/Mitosporic *Sporidiobolales* order) and Sacch (*Debaryomyces*, *Pichia*/*Saccharomycetales* order). These probes revealed a strong specificity for the target organisms. The optimal conditions for the *in situ* hybridization protocol used 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 (*R. exoculata*) and mussels (*B. azoricus*) were fixed for FISH experiments directly after dissection. The pieces of shrimps and mussels from which the highest number of fungi were isolated (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 mL^{-1} 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. A small quantity of yeast cells could be visualized on these membrane filters (Fig. 2). Such results provide further evidence of the presence of yeast cells in hydrothermal vents, albeit at low concentrations. Using FISH on membrane filters, the yeast cells detected were affiliated to three 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 & Sampaio, 2005). Yeast isolation was successful, although the retrieved species richness was relatively low. Thirty-two strains were isolated mostly from *R. exoculata* shrimps. The association with shrimps is probably favorable for yeasts, which can 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 & Sampaio, 2005). The Rainbow hydrothermal field hosted in ultramafic rocks is a unique vent rich in CH_4, H_2, CO and Fe, and deficient in H_2S (Charlou *et al.*, 2002). The high yeast isolation ratio may indicate that yeasts thrive in hydrothermal sites depleted of H_2S. The isolation rate of nonpigmented yeasts on sulfur-free media was significantly higher than those on sulfur-based media in a previous study (Gadanho & Sampaio, 2005), which supports such a hypothesis.

Several yeasts were also isolated from mussels, and more precisely from the byssus, which is composed 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.

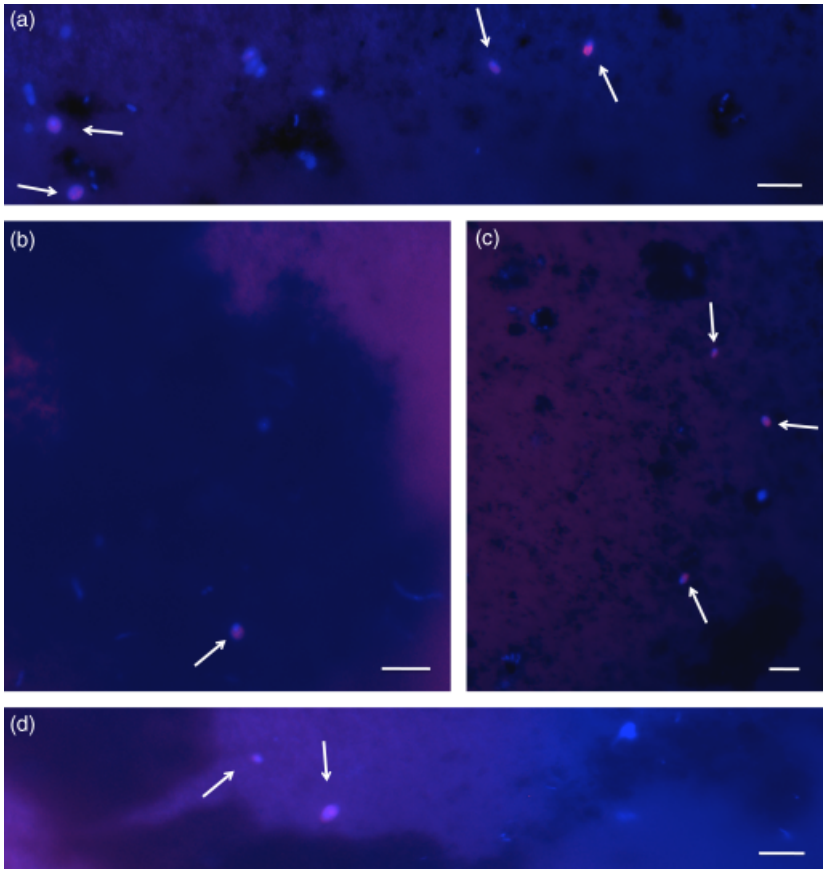

Fig. 2. FISH with specific oligonucleotide probes on membrane filters. (a–c) Membrane filter labelled with DAPI and hybridized using MitoSporidio probe indicating the presence of bacteria and yeast cells (blue). Yeasts belonging to *Rhodotorula* and *Rhodosporidium* genera are vizualized in pink (composite 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.

Pattern of the culturable yeast communities

New species

The yeast that was first 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 region of the rRNA gene, this strain was reevaluated as *Candida* sp. (Nguyen *et al*., 2009). This strain is identical to another isolated from deep-sea hydrothermal vent waters and annotated MARY089 (Gadanho & 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 is therefore supposed to be an autochthonous marine yeast species. This new ecotype can be characterized as an obligate marine yeast and its complete description is currently being investigated.

Known species

Two strains (Mo25 and Mo31) isolated from *R. exoculata* and *B. azoricus* samples were identified as *C. atlantica*. This result seems in keeping with previous published studies 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 & Sampaio, 2005). The very first *C. atlantica* strain was isolated from shrimp eggs in the North Atlantic Ocean (Siepmann & Höhnk, 1962). *Candida atlantica* appeared to be a marine obligate yeast and some interactions with shrimps seemed to occur. Our physiological analysis revealed that Mo25 and Mo31 are nonhalophiles. This 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 the growth conditions are not optimal. One isolate (Bio1) isolated from seawater surrounding mussels at Lau Basin in the South-west Pacific was clearly identified as *C. viswanathii*. Kohlmeyer & Kohlmeyer (1979) characterized this yeast as marine facultative. More recently, *C. viswanathii* was isolated from a shrimp (*Penaeus braziliensis*) in the Gulf of Mexico. Under the synonym *Candida lodderae*, this yeast was recently reported in deep-sea hydrothermal vent waters at Rainbow site (Gadanho & Sampaio, 2005) and characterized as the most abundant yeast.

Leucosporidium scottii isolates (Mo36) have been retrieved only in the oceanic regions close to Antarctica and are known to be psychrophilic and probably autochthonous marine species (Lachance & Starmer, 1998). Such strains, which are known for their presence in cold polar marine environments, could be further evidence 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 has frequently been isolated from hypersaline waters of solar salterns. In a molecular survey, *H. werneckii* was identified (based on internal transcribed spacers and 5.8S rRNA gene) in deep-sea methane seep sediments at a depth of 2965 m (Lai *et al*., 2007). *Phaeotheca triangularis* (Mo30) has also frequently been isolated from salt environments (Gunde-Cimerman *et al*., 2000) and characterized as halophilic. 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 salt optimal concentration and thus hypothesized to be a marine-adapted yeast. This is the first report of the presence of *P. triangularis* at deep-sea vents.

Mo22 is described as *S. roseus*. The genus *Sporobolomyces* is composed of strains mainly isolated from the phyllophane (Bai *et al*., 2002). However, a previous study has shown that strains of the genus *Sporobolomyces* are frequently isolated from marine ecosystems, with the frequency of isolation increasing with increasing distance from the coastline and depth (Hernandez-Saavedra *et al*., 1992). Moreover, yeasts from this genus were found in benthic invertebrates collected from the 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 composed of ubiquitous and endemic species. Typical ubiquitous strains were the ascomycetous yeast *D. hansenii* and the basidiomycetous yeasts *Cryptococcus* and *Rhodotorula*. Kohlmeyer & Kohlmeyer (1979) confirmed this statement and characterized these genera as mainly 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 & Sampaio, 2005), deep-sea sediments (Nagahama *et al*., 2001b), coastal waters (Gadanho *et al*., 2003; Gadanho & Sampaio, 2004) and oligotrophic lakes (Libkind *et al*., 2003). Our results confirm their ubiquity and indicate that these strains seem to be allochthonous. Strain Ex15 identified as *P. guilliermondii* has also been characterized as nonhalophilic and may be another allochthonous yeast strain, as reported by Kohlmeyer & Kohlmeyer (1979).

The members of the genus *Rhodosporidium* have been characterized as nonhalophiles (Mo24 and Mo33) and halotolerant (Mo38). Based on previous reports, this genus seemed to be restricted to marine environments (Gadanho & Sampaio, 2005). *Rhodosporidium 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 the carbon cycle in deep-sea environments.

Adaptation to marine conditions

The isolation of culturable yeasts has led to an old question about marine yeasts: 'Are there any indigenous marine yeasts?' (Kohlmeyer & Kohlmeyer, 1979) and to the consequent 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 nonhalophiles, 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 seawater (Kohlmeyer & Kohlmeyer, 1979). Few yeast species with a physiological dependence on NaCl or other seawater components have been reported (Nagahama, 2006). Thus, our results appear to be in good agreement with such statements. Only two strains described as halophiles (Mo34 and Mo39) in our study can be described as obligate marine yeasts.

FISH observations

FISH using labelled 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 & Sampaio, 2005), it seems that yeasts at deep-sea vents represent a minor community that may 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-labelled probe EUB338 were found to be approximately 370 16S rRNA gene molecules per cell for *Escherichia coli* hybridized on glass microscope slides and 1400 16S rRNA gene 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, etc.). Our attempts to cultivate the yeast strains in this study at elevated hydrostatic pressure have been successful, but ribosomal activities were lower at high hydrostatic pressure than at atmospheric pressure. Such results may account for the low fungal detection using FISH (G. Burgaud, unpublished data). Consequently, when using FISH alone, care must be taken when dealing with diversity and biomass estimations.

The quantification of yeasts using FISH has been impossible due to a nonhomogeneous 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 few cells visualized after filtration of seawater surrounding shrimps. This result is in keeping with the relatively low diversity revealed by Gadanho & Sampaio (2005), ranging from 0 to 10 CFU L^{-1} for pink yeasts and from 0 to 6000 CFU L^{-1} for nonpigmented 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 new questions regarding the ecological role of such microorganisms in deep-sea vents as well as 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 has already been hypothesized in previous work (Van Dover *et al.*, 2007; Burgaud *et al.*, 2009). However, they may also play a role in the decomposition of abundant organic material.

Considering all the results obtained, we can say that yeasts appear to interact with deep-sea hydrothermal endemic fauna even at low densities. 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 ubiquitous 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 done 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 difficult, with no clear answers. The culture of these yeasts 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 comments on the refinement of this manuscript. We also thank ANR Deep-Oases, Ifremer, Region Bretagne and French Research Ministry for their financial support. We finally thank Françoise Gaill and the CHEMECO/European Science Foundation EURODEEP for discussions and financial support.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Target sites of the fluorescent oligonucleotide probes designed on a model of the *Saccharomyces cerevisiae* 26S rRNA gene secondary structure in which the D1 and D2 domains (delimited by NL1 and NL4) are enlarged (Inacio *et al*., 2003).

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