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Effects of hydrostatic pressure on yeasts isolated from deep-sea hydrothermal vents

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Abstract

Hydrostatic pressure plays a significant role in the distribution of life in the biosphere. Knowledge of deep-sea piezotolerant and (hyper) piezophilic bacteria and archaea diversity has been well documented, along with their specific adaptations to cope with high hydrostatic pressure (HHP). Recent investigations of deep-sea microbial community compositions have shown unexpected micro-eukaryotic communities, mainly dominated by fungi. Molecular methods such as next-generation sequencing have been used for SSU rRNA gene sequencing to reveal fungal taxa. Currently, a difficult but fascinating challenge for marine mycologists is to create deep-sea marine fungus culture collections and assess their ability to cope with pressure. Indeed, although there is no universal genetic marker for piezoresistance, physiological analyses provide concrete relevant data for estimating their adaptations and understanding the role of fungal communities in the abyss. The present study investigated morphological and physiological responses of fungi to HHP using a collection of deep-sea yeasts as a model. The aim was to determine whether deep-sea yeasts were able to tolerate different HHP and if they were metabolically active. Here we report an unexpected taxonomic-based dichotomic response to pressure with piezosensitve ascomycetes and piezotolerant basidiomycetes, and distinct morphological switches triggered by pressure for certain strains.

Keywords: Yeasts; Marine; Hydrostatic pressure; Filamentation; Dimorphism; Piezotolerance

1. Introduction

The marine biome is considered to be the largest in the world, covering three-fourths of the Earth's surface. The average depth of the marine biome is 3800 m [1], indicating, as a Gaussian distribution, that most of the biosphere is subjected to a pressure of 38 megapascals (MPa), i.e. 380-fold higher than atmospheric pressure (0.1 MPa). Many factors regulate the biodiversity encountered in oceans, but hydrostatic pressure appears to be a key physical parameter in the dark cold abyss [2]. Deep-sea microorganisms have been classified by their cardinal growth pressures into the following categories: piezosensitive, piezotolerant, piezophiles or hyperpiezophiles [3−5]. The absolute record for the ability to grow at very high hydrostatic pressure is currently held by *Pyrococcus yayanosii*, an obligate piezophilic hyperthermophilic archaeon isolated from a deep-sea hydrothermal vent,

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with growth recorded from 20 to 120 MPa [6,7]. Such results have highlighted that hydrostatic pressure plays a significant role in prokaryotic life distribution within the deep-sea.

Recent studies have shown that deep-sea microbial communities are composed of a "not only prokaryotic" world. Indeed, numerous DNA-based studies have shown fungal communities to be present in several deep-sea ecosystems, i.e. deep sediments [8–12], hydrothermal vents [13–16], sunken woods [17], cold seeps [18,19] and even deep hypersaline anoxic basins [20–22]. More recently, next-generation sequencing technologies have led to rRNA and mRNA-based approaches to reveal: (i) metabolically active parts of these communities [10,12], and (ii) their important ecological roles, e.g. in organic carbon recycling in deep subseafloor sediments or their interactions with prokaryotes and antibiotic defense mechanisms [23]. Sequences recovered in these datasets were mainly affiliated with *Dikarya*, a subkingdom embracing the two Ascomycota and Basidiomycota phyla. To a lesser extent, some signatures of the basal *Chytridiomycota* and *Cryptomycota* phyla were also obtained. However, as such studies are based on molecular signatures, it is almost impossible to know whether fungi represented by these nucleotidic sequences are specifically adapted to deep-sea habitats. In this context, culture-based methods can be used to isolate deep-sea fungal strains and then to evaluate their ability to tolerate HHP. This will expand our knowledge on deep-sea fungi activities in situ and their ecological roles in such extreme environments.

High pressure effects on biological processes are diverse, with (i) pressure-sensitive lipids modifying fluidity, permeability and cell membrane functions, (ii) pressure-sensitive proteins affecting multimer associations and stability, thus impacting motility and cell division, and (iii) pressure-stabilized DNA hydrogen bonds affecting replication and transcription steps [24]. The impact of HHP on cell physiology has been extensively studied in the model yeast *Saccharomyces cerevisiae*. *S. cerevisiae* is piezosensitive, meaning that HHP is perceived as a stress and induces cell stress responses. The ability of *S. cerevisiae* cells to grow at moderate pressure, between 15 and 25 MPa, was shown to be directly connected to tryptophan auxotrophy and the availability of this amino acid. Cell growth arrest occurred at pressures above 50 MPa regardless of the amino acid auxotrophy of the strain. Pressures ranging from 100 to 200 MPa killed *S. cerevisiae* cells by disrupting microtubule ultrastructures, actin filaments or nuclear membranes [25]. Pressures greater than 200 MPa caused leakage of internal substrates and ions from cells. HHP also induced cytoplasmic petite mutations and tetraploid or homozygous diploid forms [1].

Few studies have specifically focused on growth of marine fungi under hydrostatic pressure. Amongst these studies, some focused on different species affiliated with the *Aspergillus* genera [26,27], while others dealt with marine yeasts such as *Rhodotorula rubra*, *Debaryomyces hansenii* and *Rhodosporidium sphaerocarpum* [28]. All studied fungal isolates were characterized as piezosensitive, as growth was systematically better at atmospheric pressure (0.1 MPa) compared to high hydrostatic pressures. A major result to be highlighted was that the 3 marine yeasts cultivated under HHP were able to grow up to at least 20 MPa, while only the basidiomycetous species *R. rubra* and *R. sphaerocarpum* were able to grow at 40 MPa, suggesting better piezotolerance of basidiomycetes compared to ascomycetes. since the analyzed strains were all ubiquitous in these studies, our goal here was to test different yeast species recently isolated from deep-sea hydrothermal vents [29], including *Candida oceani*, a novel obligate marine yeast [30]. Yeasts isolated in these studies appear well adapted to deep-sea marine conditions based on ecophysiological parameters (temperature, salinity) and the fact that some were directly observed on hydrothermal samples using fluorescent in situ hybridization.

The aim of our study was to resolve several questions of ecological interest: (i) are yeast strains able to tolerate hydrostatic pressure encountered at their isolation site,; (ii) are strains considered piezosensitive, piezotolerant or piezophile; and (iii) are strains metabolically active under elevated HHP?

2. Materials and methods

2.1. Selected strains

A collection of deep-sea yeasts was previously created from hydrothermal vent samples [29]. Amongst the 32 isolated yeasts, 21 were identified as basidiomycetes and represented by 5 different species, while 11 were defined as ascomycetes and belonged to 7 different species. For each taxonomic group, one representative isolate was selected to evaluate growth under HHP (Table 1). All isolates are available in the UBO Culture Collection (http://www.univ-brest.fr/ubocc).

2.2. Cultivation under elevated hydrostatic pressure

Yeasts were cultured under HHP in batch using a simple system based on steel cylinders filled with hydraulic fluids as previously described [31]. Yeast cells were grown in GYPS broth (0.1% glucose, 0.1% yeast extract, 0.1% peptone, 0.1% starch and 3% sea salts) at 25 °C for 24–48 h. Exponentially growing cells were then resuspended in GYPS broth to a concentration of about 1.10^6 cells/ml and placed in 6 ml plastic tubes (Nunc© cryotubes) in triplicate under oxic conditions. After sealing with sterile parafilm, tubes were placed in pressure vessels filled with sterile water (used as the hydraulic fluid) and subjected to hydrostatic pressure [32]. The required hydrostatic pressures, i.e. 7 MPa (only for *Pichia guilliermondii* Ex15), 24.5 MPa, 60 MPa and 0.1 MPa (control condition, also incubated in HP tubes), were reached in less than 2 min using a hand-pump. Pressure vessels were then incubated at 25 °C for 96 h.

After incubation, pressure was released in approximately 15 s. Plastic tubes were immediately placed on ice. Then, the content of each tube was directly used for:(i) biomass quantification, (ii) yeast cell ribosomal activity estimate coupled with DAPI and calcofluor staining; and (iii) morphological analyses.

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Table 1
Characteristics of the selected deep-sea yeasts.

ID	Genus	Species	Depth (m)	Phylum	Order	UBOCC number
Mo22	*Sporobolomyces*	*roseus*	2300 m	Basidiomycota	Sporidiobolales	UBOCC-A-208018
Mo26	*Cryptococcus*	sp.	2300 m	Basidiomycota	Filobasidiales	UBOCC-A-208021
Mo32	*Rhodotorula*	*mucilaginosa*	2300 m	Basidiomycota	Sporidiobolales	UBOCC-A-208027
Mo38	*Rhodosporidium*	*diobovatum*	2300 m	Basidiomycota	Sporidiobolales	UBOCC-A-208033
Bio1	*Candida*	*viswanathii*	2620	Ascomycota	Saccharomycetales	UBOCC-A-208001
Bio2	*Debaryomyces*	*hansenii*	2620	Ascomycota	Saccharomycetales	UBOCC-A-208002
Mo40	*Debaryomyces*	*hansenii*	2300	Ascomycota	Saccharomycetales	UBOCC-A-208035
Ex15	*Pichia*	*guilliermondii*	700	Ascomycota	Saccharomycetales	UBOCC-A-208004
Mo30	*Phaeotheca*	*triangularis*	2300	Ascomycota	mitosporic Ascomycota	UBOCC-A-208025
Mo31	*Candida*	*atlantica*	2300	Ascomycota	Saccharomycetales	UBOCC-A-208026
Mo34	*Hortaea*	*werneckii*	2300	Ascomycota	Dothideales	UBOCC-A-208029
Mo39	*Candida*	*oceani*	2300	Ascomycota	Saccharomycetales	UBOCC-A-208034

2.3. Biomass quantification

After verifying purity (i.e. absence of bacterial contaminants), optical density (OD) and total ergosterol content were determined as indicators of biomass on three biological replicates: (i) OD at 600 nm was measured using a Helios spectrophotometer (Thermo Scientific); (ii) total ergosterol was extracted and quantified as previously described [33,34]. Measures were compared to starting indicator values of each culture used as a control. Each culture had a starting OD value of 0.1, representing 1.10^6 cells/ml, and about 0.5 µg/ml total ergosterol concentration.

Prior to ergosterol quantification by high performance liquid chromatography (HPLC), samples were resuspended in 0.5 mL 100% methanol, homogenized and filtered through a 0.45 µm filter into glass vials protected from light. HPLC was performed using a 50 µl injection loop, a flow rate for methanol set to 1.2 ml/min and a ZORBAX Eclipse XDB-C18 4.6 × 150 mm−5 microns column. Ergosterol was detected at 282 nm with a 7.8 min retention time. The linear relationship between peak area and concentration was determined using ergosterol standard solutions (0.2 µg/ml to 10 µg/ml) injected before each analysis set.

2.4. Fluorescent in situ hybridization, DAPI and calcofluor staining

For each replicate, 1 ml of culture was fixed in 4% paraformaldehyde for 3 h at 4 °C. Cells were washed by centrifugation (8000 g, 4 °C, 10 min) with phosphate buffered saline solution (1X PBS, pH 7.4). Fixed cells were resuspended in 1X PBS/96° ethanol and stored at −20 °C until analysis. rRNA hybridization was processed on a 10-well Teflon-coated slides as previously described [29] using the universal eukaryotic probe Euk516 labeled with Cy3. Slides were also DAPI-stained (final concentration 1 µg/ml) and mounted with antifading reagent Citifluor AF 2 (Citifluor, France) before observation under a fluorescent microscope. Calcofluor white staining was also used to examine fungal cells on the same slides. Slides were stained with 0.5 mM calcofluor white M2R solution (Sigma Aldrich, St. Louis, MO, USA) to target chitin, cellulose and carboxylated polysaccharides. Following incubation in the dark for 5 min, slides were washed with sterile water and observed using epifluorescence microscopy. Hybridized cells were observed with a 100X oil immersion objective using epifluorescence microscopy (Olympus). Resulting images were recorded as 600 dpi.tiff files with the same acquisition time (200 ms). Hybridized cell images were analyzed using DAIME [35] that allows calculating mean fluorescence cell intensity (mean of 9 repetitions for each sample).

2.5. Morphological analyses

Yeast cells were observed using light microscopy (Olympus) and scanning electron microscopy (SEM) with an XL 30 LaB6 apparatus (Philips). For SEM, cells were concentrated on 0.22 µm Nuclepore filters and dried overnight at room temperature with formaldehyde vapors. Filters were coated with gold using a Balzers Ions Sputter SCD-040.

2.6. Microscopy under pressure

Yeast cells were diluted to a concentration of about 10^6 cells/mL. An aliquot of 1 ml was deposited between 2 sapphire windows of an original optical cell designed and built by Top'industrie (France) (2 sapphire window thickness was 3.5 mm, usable visualization volume less than 0.1 ml, max pressure 400 MPa, ambient temperature). The optical reactor was adapted from a previous observation device [36].

Yeast cells were observed through the optical reactor using an inverted microscope (Nikon Eclipse TE 2000 U, Nikon, Japan) equipped with a color camera (Nikon Digital Sight DS-U1). A 25X objective wide distance (Leica) was used to observe cells and images were snapped every 18 min (frequency 0.001 Hz). In order to avoid continuous highlighting due to the photonic lamp, a shutter was added. Acquired images were analyzed using ImageJ (1.43u, NIH, USA).

3. Results

3.1. Ability of deep-sea yeasts to tolerate elevated hydrostatic pressure

The first aim of this study was to assess yeast species response to different HHP. Two markers were used for

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biomass quantification, namely OD at 600 nm and total ergosterol content. Yeasts were cultured under at least 3 different hydrostatic pressures: (i) 0.1 MPa, atmospheric pressure (control condition); (ii) 7 and/or 24.5 MPa, corresponding to sampling site hydrostatic pressure (Table 1); and (iii) 60 MPa for strain classification as piezosensitive, piezotolerant or piezophilic.

The eight selected ascomycete yeasts showed significantly similar response patterns for both biomass indicators (Table 2). Higher biomass quantities were measured for all strains at atmospheric pressure (0.1 MPa) after 96 h incubation. At 24.5 MPa hydrostatic pressure, 5 ascomycete yeasts, namely *Candida viswanathii* (Bio1), *D. hansenii* (Bio2 and Mo40), *P. guilliermondii* (Ex15) and *P. triangularis* (Mo30), appeared to better tolerate this pressure compared to *Candida atlantica* (Mo31), *Hortaea werneckii* (Mo34) and *C. oceani* (Mo39). Indeed, after 96 h, biomass quantities increased, although these values were approximately 30–70% lower at 24.5 MPa compared to control conditions (0.1 MPa). Furthermore, except for *P. triangularis*, no ascomycetes resisted 60 MPa. Both OD and ergosterol values were below the original inoculum (<0.1 OD 600 nm and <0.5 μg/ml ergosterol). These results indicate that the studied deep-sea ascomycetous yeasts are pressure-sensitive, e.g. piezosensitive.

Basidiomycete yeasts (n = 4) revealed 2 different response patterns (Table 3). Two strains were more tolerant to HHP (*Rothia mucilaginosa*, Mo32 and *Rhodosporidium diobovatum*, Mo38), with almost no variations in biomass content at 0.1 and 24.5 MPa, while *Saccharomyces roseus* (Mo22) *and Cryptococcus* sp. (Mo26) biomass contents clearly decreased at 24.5 MPa after 96 h incubation. At both tested hydrostatic pressures (24.5 and 60 MPa), biomass measurements were similar for Mo32 and Mo38; noteworthy, slightly higher biomass was actually observed for Mo32 at 24.5 MPa (Table 3). However, taking into account standard deviations, no statistical differences in biomass contents were observed between 0.1 and 24.5 MPa. Interestingly, compared to ascomycetes, lower decreases in biomass contents were observed under HHP, as high biomass measurements were obtained at both 0.1 and 24.5 MPa. Moreover, all basidiomycetes were capable of resisting 60 MPa hydrostatic pressure, as OD and ergosterol values were both higher than those of the original inoculum.

For both ascomycetes and basidiomycetes, the selected biomass markers gave similar patterns, indicating that both were suitable for our study. The tested conditions showed that deep-sea basidiomycete yeasts are clearly less pressure-sensitive microorganisms compared to ascomycetes and may be classified as piezotolerant.

3.2. Ribosomal activity under elevated hydrostatic pressure

Fluorescent probe hybridization to ribosomal RNA (rRNA) allowed obtaining a signal correlated with ribosome content in cells and thus protein activity [37]. This parameter was used as an indicator of yeast strain physiological activity. Ribosomal fluorescence intensity was thus quantified as a proxy for cellular activity (Tables 4 and 5). This analysis showed 3 different response types to HHP:

(i) Minor or no significant differences at all pressures, i.e. 0.1, 24.5 and 60 MPa, for 5 ascomycetes (Bio1, Bio2, Mo39, Mo40 and Ex15) and 2 basidiomycetes (Mo32 and Mo38)
(ii) A decrease in fluorescence signal for protein activities observed at 60 MPa for 2 ascomycetes (Mo31 and Mo34) and 2 basidiomycetes (Mo22 and Mo26)

3.3. A decrease in fluorescence signal and no detected protein activity at 60 MPa for 1 ascomycete (Mo30)

3.3.1. Morphological switch under elevated hydrostatic pressure

Yeast cell observations were performed by light microscopy for all species at all studied hydrostatic pressures. In most cases, no morphological differences were seen between cells cultured at 0.1 MPa and those at 7 or 24.5 MPa.

Table 2
Ability of ascomycete yeasts to tolerate HHP using optical density (OD 600 nm) and total ergosterol concentration measurements (μg/ml).

ID	Species	Indicator	0.1 MPa	7 MPa	24.5 MPa	60 MPa
Bio1	*C. viswanathii*	OD	0.273 ± 0.016		0.134 ± 0.010	0.016 ± 0.019
		Ergosterol	1.419 ± 0.038		0.703 ± 0.025	0.220 ± 0.018
Bio2	*D. hansenii*	OD	0.359 ± 0.008		0.193 ± 0.021	0.070 ± 0.004
		Ergosterol	0.896 ± 0.225		0.551 ± 0.084	0.215 ± 0.072
Mo40	*D. hansenii*	OD	0.446 ± 0.016		0.249 ± 0.032	0.064 ± 0.006
		Ergosterol	1.275 ± 0.035		0.829 ± 0.021	0.401 ± 0.011
Ex15	*P. guilliermondii*	OD	0.634 ± 0.029	0.538 ± 0.050	0.220 ± 0.004	0.024 ± 0.004
		Ergosterol	1.729 ± 0.053	1.462 ± 0.054	0.641 ± 0.030	0.428 ± 0.018
Mo30	*P. triangularis*	OD	0.264 ± 0.020		0.194 ± 0.032	0.139 ± 0.017
		Ergosterol	1.513 ± 0.030		0.968 ± 0.005	0.817 ± 0.004
Mo31	*C. atlantica*	OD	0.254 ± 0.028		0.074 ± 0.011	0.015 ± 0.003
		Ergosterol	1.334 ± 0.052		0.578 ± 0.087	0.393 ± 0.010
Mo34	*H. werneckii*	OD	0.190 ± 0.027		0.092 ± 0.006	0.059 ± 0.009
		Ergosterol	1.262 ± 0.171		0.860 ± 0.067	0.648 ± 0.070
Mo39	*C. oceani*	OD	0.212 ± 0.009		0.087 ± 0.003	0.071 ± 0.002
		Ergosterol	1.814 ± 0.004		0.449 ± 0.050	0.330 ± 0.104

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Table 3
Ability of basidiomycete yeasts to tolerate HHP using optical density (OD 600 nm) and total ergosterol concentration measurements (μg/ml).

ID	Species	Indicator	0.1 MPa	24.5 MPa	60 MPa
Mo22	*S. roseus*	OD	0.170 ± 0.012	0.127 ± 0.08	0.088 ± 0.002
		Ergosterol	1.482 ± 0.317	0.941 ± 0.135	0.984 ± 0.109
Mo26	*Cryptococcus* sp.	OD	0.336 ± 0.020	0.268 ± 0.003	0.211 ± 0.004
		Ergosterol	1.439 ± 0.092	1.097 ± 0.141	0.995 ± 0.107
Mo32	*R. mucilaginosa*	OD	0.150 ± 0.013	0.175 ± 0.013	0.127 ± 0.003
		Ergosterol	0.801 ± 0.133	0.915 ± 0.155	0.343 ± 0.219
Mo38	*R. diobovatum*	OD	0.324 ± 0.007	0.316 ± 0.011	0.166 ± 0.003
		Ergosterol	2.251 ± 0.211	2.173 ± 0.250	1.260 ± 0.063

Table 4
Mean fluorescence intensity of ascomycete cells under HHP (confidence interval of the mean at 95% based on 9 replicates).

ID	Species	0.1 MPa	7 MPa	24.5 MPa	60 MPa
Bio1	*C. viswanathii*	124.8 ± 7.0		111.0 ± 2.3	111.9 ± 8.2
Bio2	*D. hansenii*	127.5 ± 1.1		114.0 ± 0.7	117.4 ± 1.1
Mo40	*D. hansenii*	116.4 ± 1.2		106.2 ± 0.9	102.1 ± 1.4
Ex15	*P. guilliermondii*	116.5 ± 6.0	109.6 ± 1.0	106.2 ± 1.8	111.1 ± 2.6
Mo30	*P. triangularis*	99.7 ± 8.5		70.6 ± 13.7	No fluorescence
Mo31	*C. atlantica*	98.2 ± 1.2		106.1 ± 2.8	62.2 ± 1.7
Mo34	*H. werneckii*	132.0 ± 0.9		114.1 ± 3.0	50.8 ± 5.7
Mo39	*C. oceani*	80.8 ± 3.4		83.9 ± 1.7	73.2 ± 1.1

Table 5
Mean fluorescence intensity of basidiomycete cells under HHP (confidence interval of the mean at 95% based on 9 replicates).

ID	Species	0.1 MPa	24.5 MPa	60 MPa
Mo22	*S. roseus*	116.1 ± 1.2	112.5 ± 1.2	64.4 ± 2.2
Mo26	*Cryptococcus* sp.	78.0 ± 1.6	61.0 ± 2.0	44.3 ± 2.2
Mo32	*R. mucilaginosa*	119.3 ± 0.8	120.2 ± 0.9	95.4 ± 1.3
Mo38	*R. diobovatum*	125.5 ± 0.6	120.1 ± 0.7	101.9 ± 1.2

Interestingly, strains belonging to 3 yeast species showed strong morphological differences at 24.5 MPa. *C. atlantica* (Mo31) and *P. guilliermondii* (Ex15) switched from unique cells to pseudohyphae, while *C. viswanathii* (Bio1) switched from unique cells to a dense network of filaments resembling hyphae as hydrostatic pressure increased (Fig. 1).

Complementary analyses were performed on strains belonging to these species to highlight their filamentation threshold. Yeasts were cultivated under different hydrostatic pressures ranging from 5 to 24.5 MPa; then, cells were hybridized with the Euk516-Cy3 probe and DAPI stained. Figs. 2, S1 and S2 clearly indicate that filamentation occurred at the lowest hydrostatic pressure tested (5 MPa) and seemed to be amplified with HHP, especially for *C. viswanathii* Bio1. Moreover, these analyses confirmed cell viability, as seen by both intense ribosomal activity and cell division, as nuclei were clearly present under HHP.

In order to affirm that Bio1 form "true" hyphae under HHP, calcofluor white staining was performed to highlight chitinous cell walls. Fig. 3 distinctly shows that *C. viswanathii* Bio1 filaments are compartmentalized by multiple septa, thus demonstrating that true hyphae are formed under HHP.

Finally, in order to prove that HHP was the only parameter inducing filamentation, microscopy under pressure was also performed on *C. viswanathii* Bio1 cells at different pressures. Fig. 4 reveals that Bio1 grows as budding yeast at 0.1 MPa and exhibits a morphological switch immediately after pressure increase.

4. Discussion

Recently, yeasts were considered dominant amongst fungi in the deep-sea, as revealed by culture-independent methods [15]. However, without any universal genetic marker for piezoresistance, culture-based analyses appear to be an interesting and complementary approach to evaluating HHP tolerance of such organisms in the deep oceans. Such analyses will also provide information for better understanding the role of deep-sea yeasts in the dark cold abyss.

4.1. Piezotolerance of deep-sea yeasts

The most ecologically relevant information obtained in this study was that all selected deep-sea hydrothermal yeasts were able to tolerate hydrostatic pressure encountered at their isolation site. This ecophysiological characterization is clearly shown here to be a crucial step in order to clearly demonstrate the ability of deep-sea fungi to grow in high-pressure extreme environments and, indirectly, to demonstrate their activity and potential ecological roles in the dark cold abyss. Ascomycetes appeared sensitive to HHP, especially at 60 MPa, where biomass was lower than the inoculum. This confirms the results of a previous study on the ascomycete yeast *S. cerevisiae* that highlighted cell division arrest at 50 MPa [38]. However,

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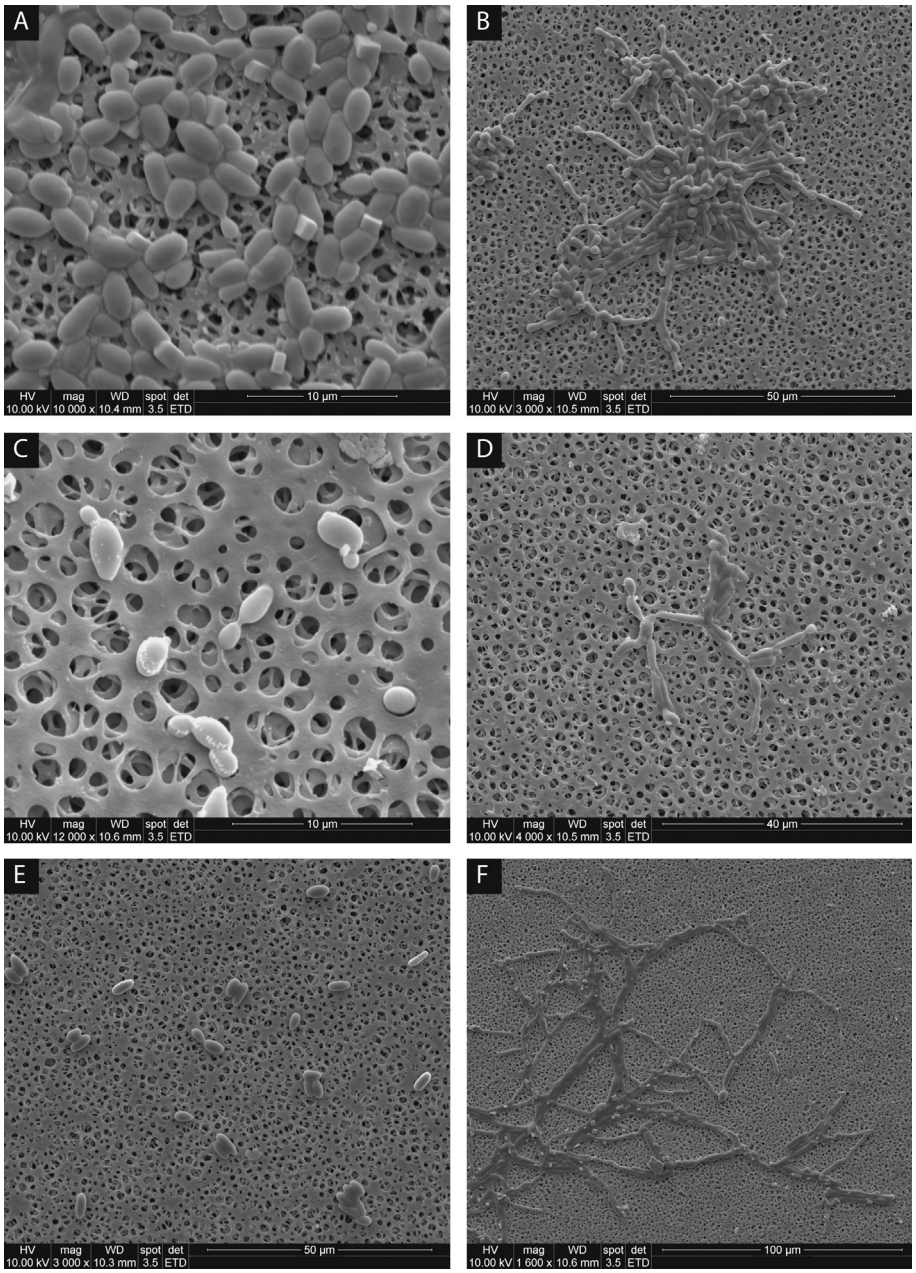

Fig. 1. Scanning electron microscopic observations of Ex15 (A–B), Mo31 (C–D) and Bio1 (E–F) morphological switches – yeast to filamentous growth – in response to HHP from 0.1 MPa (A–C–E) to 24.5 MPa (B, D, F).

in our case, ribosomal activity was still observed at 60 MPa (except for *P. triangularis,* Mo30), and tends to confirm another study demonstrating *S. cerevisiae* glucose fermentation capacity at pressures up to 87 MPa [39]. Protein activity quantification by ribosomal rRNA content evaluation appears to be a complementary method for assessing the yeast response to HHP. Indeed, if all ascomycetes are able to tolerate 24.5 MPa hydrostatic pressure, some appear less impacted by HHP than others, as was observed for *C. viswanathii* (Bio1), *D. hansenii* (Bio2 and Mo40), *C. oceani* (Mo39) and *P. guilliermondii* (Ex15) when compared to *C. atlantica* (Mo31) and *H. werneckii* (Mo34). This method also showed that *P. triangularis* (Mo30) is a true piezosensitive yeast, as no FISH signal was observed at 60 MPa, clearly indicating that Mo30 cells are inactivated or destroyed at the highest hydrostatic pressure condition tested.

Basidiomycetes clearly appeared less impacted by HHP, particularly *R. mucilaginosa* (Mo32) and *R. diobovatum* (Mo38). rRNA content quantification confirmed these results, as Mo32 and Mo38 appeared less sensitive to HHP than *S. roseus* (Mo22) and Cryptococcus sp. (Mo26). Our results appear consistent with a previous study on 3 marine yeasts (2 basidiomycetes and 1 ascomycete) revealing that basidiomycetes were less impacted by 40 MPa hydrostatic pressure compared to ascomycetes [28]. Another study already highlighted *Rhodotorula glutinis* piezotolerance based on the effect

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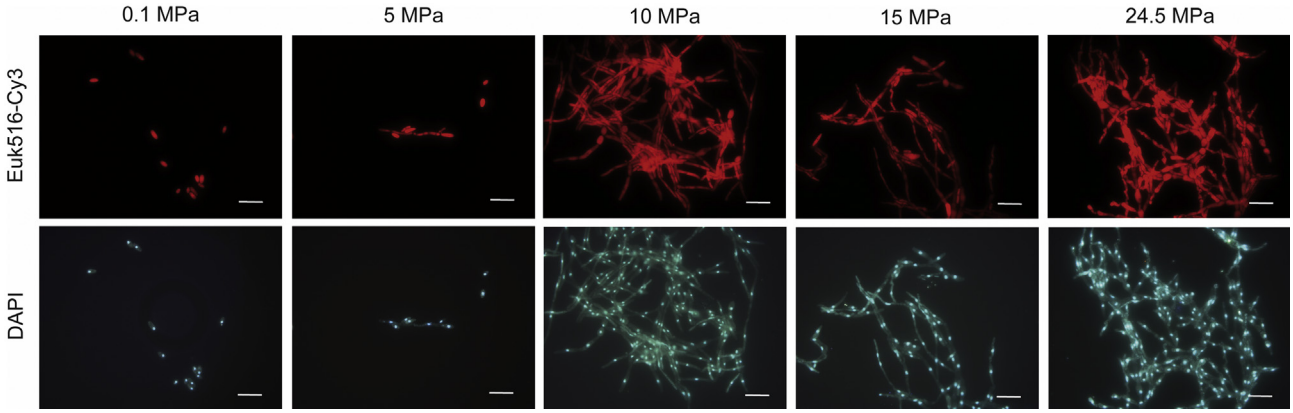

Fig. 2. Cellular activity and cell division of *Candida viswanathii* (Bio1) under different HHP, as shown by Euk516 hybridization and DAPI staining after 96 h incubation (Scale bar: 10 μm).

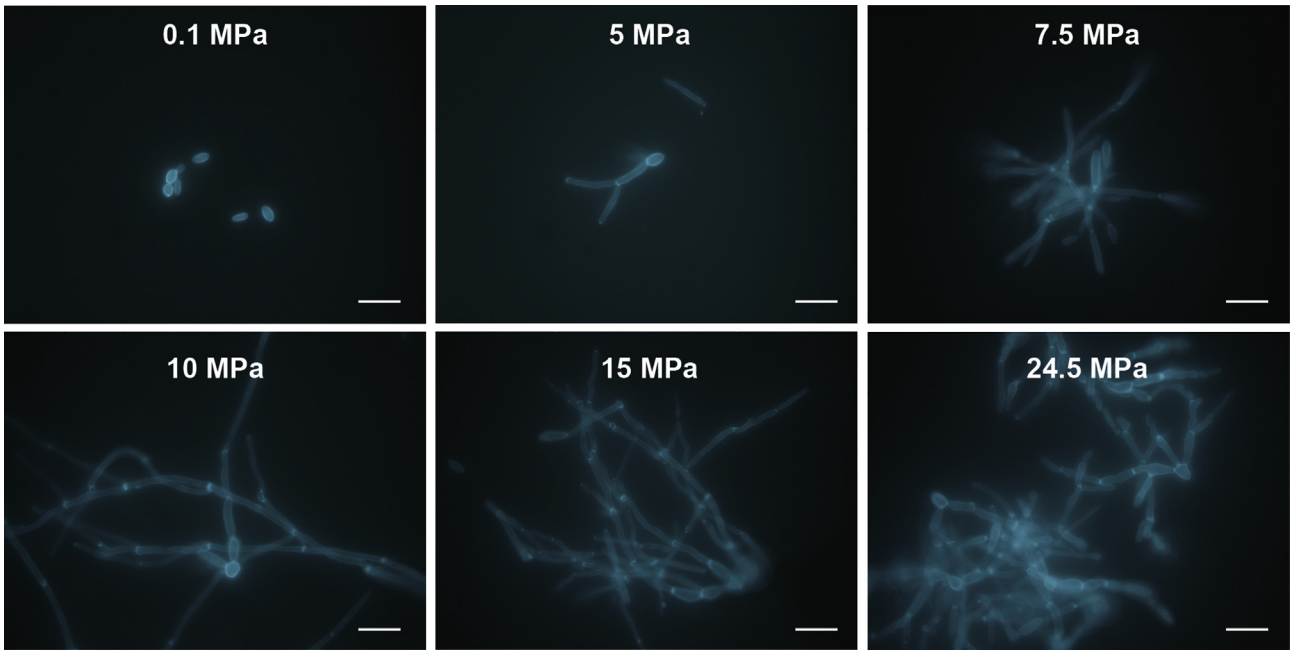

Fig. 3. Calcofluor white staining of *C. viswanathii* (Bio1) cultured at different HHP indicating fungal septa after 96 h incubation (Scale bar: 10 μm).

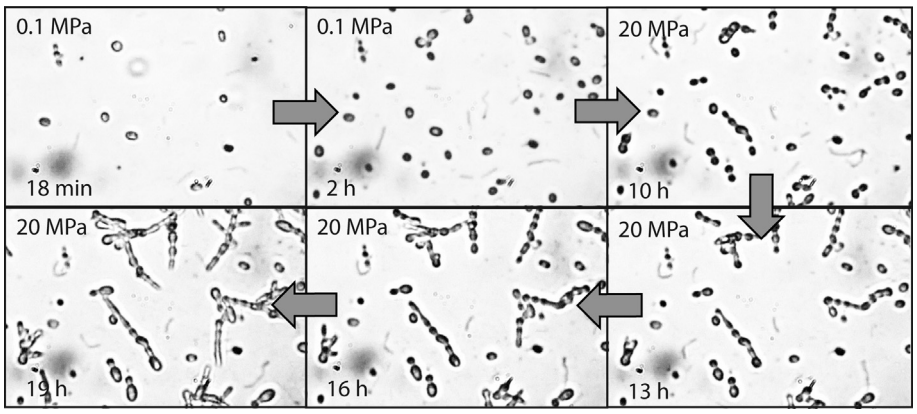

Fig. 4. Morphological switch of *C. viswanathii* (Bio1) under HHP as revealed by microscopy under pressure (movie strip showing yeast filamentation after 10 h growth, induced by HHP).

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of high hydrostatic pressure stress on cells, i.e. a 300 MPa treatment for 15 min [40].

R. mucilaginosa and *R. diovobatum* have both been isolated from different kinds of deep-sea marine ecosystems. *R. mucilaginosa* sequences have already been detected in deep seawater samples at 3800 and 4970 m [15], while *R. mucilaginosa* strains have been isolated from deep seawater samples surrounding the Rainbow hydrothermal site in the Mid-Atlantic ridge at 2300 m [41] and deep-sea mussels and shrimp samples at the same hydrothermal site [29]. *R. mucilaginosa* has been defined as a ubiquitous yeast species occurring in marine and aquatic environments. Such ubiquity may be related to its capacity to tolerate numerous stresses, including HHP. For *R. diobovatum*, sequences have also been recovered in deep seawater samples at 3000 m and bacterial mats at 1575 m [15]. *R. diobovatum* strains have also been isolated from deep seawater surrounding the Rainbow hydrothermal site in the Mid-Atlantic ridge at 2300 m [41] and deep-sea mussels, shrimp and sponge samples at the same hydrothermal site [29]. This species is mostly isolated from marine and estuarian habitats, and has previously been characterized as a marine ecotype [29,41]. In this study, its classification as piezotolerant confirms this statement and clearly highlights its capacity to grow and thus to have an ecological role in deep-sea environments.

Recently, Burgaud et al. [29] used fluorescent in situ hybridization with specific probes and observed active basidiomycete yeasts from deep-sea hydrothermal samples, whereas no ascomycete yeast cells were ever visualized. Our results appeared consistent with such observations, in that basidiomycetes seem more prevalent in deep-sea environments. This is most likely due to their piezotolerance. On the contrary, ascomycetes presence may be more limited, i.e. below the detection limit of the FISH technique, probably due to their piezosensitivity.

Ecophysiological characterization using HHP provides complementary information for assessing the ability of microorganisms to grow in deep-sea ecosystems where hydrostatic pressure is the main physical parameter [2]. Here, our results demonstrate a clear taxonomic-based dichotomic response to HHP between ascomycetes and basidiomycetes. Such results are of interest, since ascomycetes are known to tolerate much more stressful conditions than basidiomycetes, e.g. low/high temperature, low/high pH, low water availability, etc. [42]. However, it could be phenomenological, as ascomycetes are much more frequently studied as model fungi than basidiomycetes. Indeed, the fact that ascomycetes and basidiomycetes are detected in different kinds of deep-sea extreme environments [8–23] tends to confirm that they are both able to tolerate HHP. However, to assess whether the degree of response to HHP is always linked to taxonomy, this analysis needs to be done using the same methodology on other deep-sea fungal species already preserved in other culture collections, so as to properly evaluate their actual ability to grow under HHP. Such approaches will enable determination of the proportion of fungal species isolated thus far that are truly able to tolerate hydrostatic pressure encountered in the deep-sea.

4.2. Piezosensitive strain dimorphism: stress or adaptation?

Among the eight ascomycete yeasts analyzed and characterized as piezosensitive, three exhibited filamentous morphology under HHP. Filamentation of microorganisms under HHP has already been observed for *Escherichia coli* when hydrostatic pressure increases from 0.1 to 40–50 MPa [43]. For yeasts, to date, no study has demonstrated such a phenomenon induced by HHP [1]. Our study revealed two unique kinds of filamentation: pseudo-hyphae were identified for *C. atlantica* (Mo31) and *P. guilliermondii* (Ex15), while true hyphae were observed for *C. viswanathii* (Bio1). These morphological switches were non-permanent, as all yeasts cultured under HHP restarted typical unicellular growth when cultured at 0.1 MPa.

In comparison, *E. coli* filamentation occurs without any septum formation when cultured at 40 MPa HHP. This represents a well-defined phenomenon due to FtsZ protein inhibition by HHP [44]. FtsZ has been shown to play a critical role in cytokinesis. Here, the question was to determine whether *C. viswanathii* (Bio1) filamentation observed under HHP was the result of a similar phenomenon involving inhibition of major components in the cytokinesis process, such as the β-tubulin protein. Our microscopic observations clearly revealed that *C. viswanathii* (Bio1) fungal filaments were partitioned by cross-wall septa and thus strongly support the idea of complete cytokinesis.

Different environmental conditions can induce a rapid switch from yeast to filamentous growth. For example, phosphate-rich media induce pseudohyphae formation in *Candida albicans* [45], while this switch can also be activated by cells subjected to relatively high temperatures (37 °C) or osmotic stress (1 M NaCl) in *S. cerevisiae*. Different kinds of stress applied to *S. cerevisiae* seem to either activate or inactivate different pathways that involve this kind of morphological change [46]. The role of the cyclic adenosine monophosphate (cAMP)-mediated pathway has been determined in the yeast-to-hyphal switch [47]. Similarly, some *C. albicans* mutants lacking functions in essential cell cycle components also lead to pseudohyphae formation [48]. Morphological switches observed for *S. cerevisiae* and *C. albicans* appear as a response to adverse growth conditions. This is consistent with our data, indicating that dimorphic deep-sea yeasts are only pressure-sensitive microorganisms.

An HHP-induced filamentation phenomenon appears to be unique. Moreover, this event is totally different from *E. coli* cytokinesis inhibition by HHP. Further studies should be performed to fully understand this original morphological switch, e.g. analyses of specific stress markers (for example, trehalose, heat shock proteins, etc.) using qPCR but also comparative genomic approaches coupled with transcriptomic analyses.

Fungal community analyses from deep oceans remain an underexplored topic. This is particularly true for culturable fungal species diversity compared to 18 S rDNA molecular surveys. This can be related to: (i) the extreme scarcity of marine mycologists interested in the deep ocean; and (ii) the

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need for suitable specific equipment to culture deep-sea microorganisms under HHP. Here, our aim was to perform an original multidisciplinary study in order to obtain original scientific results on the ability of deep-sea fungal strains to grow under HHP. Our in-depth analysis compiles biomass quantification data obtained using different markers to infer the impact of HHP on cells and their physiology by determining rRNA content and scanning electron microscopy and microscopy under pressure observations. Our main results revealed that: all targeted yeasts previously isolated from deep-sea hydrothermal vents, are able to grow under HHP; (ii) some yeasts appear better adapted to increasing HHP; and (iii) some piezosensitive yeasts display a unique and complex morphological switch induced by hydrostatic pressure. Finally, the presence of ubiquitous adapted yeasts in the deep oceans highlights the need to further elucidate the analysis process and carry out comparative genomic analyses with terrestrial representatives that will undoubtedly determine adaptation traits of yeasts under deep-sea conditions.

Conflict of interest

No conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.resmic.2015.07.005.

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