Microbial Ecology

Occurrence and Diversity of Yeasts in the Mid-Atlantic Ridge Hydrothermal Fields Near the Azores Archipelago

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Received: 30 September 2004 / Accepted: 11 February 2005 / Online publication: 24 November 2005

Abstract

The yeast community associated with deep-sea hydrothermal systems of the Mid-Atlantic Rift was surveyed for the first time. This study relied on a culture-based approach using two different growth media: a conventional culture medium for yeasts supplemented with sea salts (MYPss) and the same medium additionally supplemented with sulfur (MYPssS). For the evaluation of species diversity, a molecular approach involving minisatellite-primed polymerase chain reaction (MSP-PCR) strain typing and sequence analysis of the D1/D2 domains of the 26S rDNA was followed. In the seven water samples that were studied, the number of colony-forming units per liter (cfu/L) ranged from 0 to 5940. The nonpigmented yeasts were much more abundant than the pink-pigmented ones. This disproportion was not observed in studies of other marine systems and may be due to the unique conditions of hydrothermal vents, characterized by a rich animal and microbial diversity and therefore by the availability of organic compounds utilizable by yeasts. Higher counts of nonpigmented yeast were obtained using MYPss, whereas for pink yeasts, higher counts were obtained using MYPssS. Moreover, among pink yeasts, some of the MSP-PCR classes obtained were composed of isolates obtained only on MYPssS, which might be an indication that these isolates are adapted to the ecosystems of the hydrothermal vents. Twelve phylotypes belonged to the Ascomycota and seven phylotypes belonged to the Basidiomycota. The nonpigmented yeasts were identified as *Candida atlantica*, *C. atmosphaerica*, *C. lodderae*, *C. parapsilosis*, *Exophiala dermatitidis*, *Pichia guilliermondii*, and *Trichosporon dermatis*, whereas the pigmented yeasts were identified as *Rhodosporidium diobovatum*, *R. sphaerocarpum*, *R. toruloides*, and *Rhodotorula mucilaginosa*. Some of the yeasts that were found belong to phylogenetic groups that include species reported from other marine environments, and eight phylotypes represent undescribed species. The new phylotypes found at Mid-Atlantic Ridge hydrothermal fields represent 33% of the total number of yeast taxa that were found.

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Introduction

The Mid-Atlantic Ridge (MAR) is a submerged mountain range, which extends from the Arctic Ocean to beyond the southern tip of Africa. In 1985, the first MAR hydrothermal field, the Trans-Atlantic Geotraverse, was discovered [20]. Since then, other hydrothermal systems have been described. The best studied are Menez Gwen, Lucky Strike, and Rainbow [12, 18]. Hydrothermal vents form when the hot and mineral-rich water flows into the ocean floor through volcanic lava. The hydrothermal emissions that come out through the black smokers can reach 300°C. The black smokers spew mostly iron and sulfide that combine to form iron monosulfide, which gives the smoker its black color. When the emanations mix with the surrounding cold water (3.5°C on average), diverse geochemical and temperature gradients are formed. Several sites along the MAR feature a high concentration of methane in the water, which is a characteristic of hydrothermal activity, but no chimneys have been detected. Two of these methane anomaly fields are Mount Saldanha and Menez Hom.

Hydrothermal vents constitute ecosystems with a rich microbial diversity that relies on chemolithoautothrophic primary production. Biogeochemical studies have elucidated the role of several prokaryotes such as the sulfur-oxidizing bacteria [8], methane-oxidizing mesophiles [21], and endosymbionts [23]. Most studies of deep-sea vents used enrichment culturing techniques for growing

 DOI: 10.1007/s00248-005-0195-y • Volume 50, 408–417 (2005)

prokaryotic hyperthermophiles [10, 19] and molecular phylogenetic approaches directed to the small-subunit rRNA gene (16S rDNA). A novel diversity bacterial and archaeal sequences were discovered [6, 17]. With respect to the microeukaryotes, the use of 18S rDNA universal primers yielded analogous results, whereby sequences of unknown Stramenopiles and Alveolata were found [1].

The occurrence of unicellular fungi, usually designated as yeasts, in deep-sea environments not associated with hydrothermal activity in the Northwest Pacific Ocean has been reported [14]. Yeasts were found on several substrates including benthic animals (shrimps, mussels, and tubeworms) and in seafloor sediment at depths ranging from 6400 to 11,000 m. Several of the yeasts isolated represented new species in the Ascomycetes and Basidiomycetes [13, 15, 16], which suggests that these unicellular eukaryotes are autochthonous microbial populations. To our knowledge, nothing is known about yeast incidence and diversity in hydrothermal fields. Presence in high-temperature locations of hydrothermal vents is not likely because fungal thermal tolerance is much lower than that of some prokaryotes, and up to now, no thermophilic yeasts are known (fungi are considered thermophilic if they grow at 50°C or higher temperatures and do not grow at 20°C or lower temperatures). However, the thermal gradient in hydrothermal fields is such that in a few centimeters, temperature can drop from 300 to 3°C. Therefore, adequate conditions for mesophiles can also be found. Moreover, because of the high density of different life forms in hydrothermal vents, it is also possible that yeasts, which can act as decomposers in more conventional ecosystems, have access to organic compounds resulting from the death of animals and microorganisms. Therefore, the availability of nutrients could support the growth of decomposers, which in turn could play a role in the recycling of organic matter.

To investigate the occurrence and diversity of yeasts in hydrothermal systems, water samples collected during the Portuguese research cruise SEAHMA-I in five MAR sites located near the Azores archipelago were used for yeast isolation. The study relied on a culture-based approach using two different growth media: a conventional culture medium for yeasts supplemented with sea salts and a similar medium further supplemented with sulfur. Because of its abundance at hydrothermal vents, elemental sulfur is normally incorporated in culture media employed for the isolation of prokaryotes [22], a strategy also adopted in the present study. The samples were processed on both media, and the influence of each medium on yeast counts and diversity was evaluated.

Materials and Methods

Sampling Sites. On August 2002, five MAR sites (Lucky Strike, Menez Gwen, Menez Hom, Mount Saldanha, and Rainbow) were visited during the Portuguese research cruise SEAHMA-1 (Seafloor and sub-Seafloor Hydrothermal Modelling in the Azores Sea) as shown in Fig. 1. Deep-sea sampling was performed on various targets along a 270-km line southwest of the Azores archipelago, using the submersible VICTOR 6000 on board of the Institut Français de Recherche pour L'Éxploitation de la Mer (IFREMER) research vessel L'Atalante. The Menez Gwen and Lucky Strike fields are located on top of neovolcanic edifices associated with basaltic lava making up the oceanic crust and are located at moderate depths (800–1000 and 1600–1750 m, respectively). By contrast, the Rainbow (2230–2500 m) and Saldanha (2100–3150 m) fields lie on faults at the edge of segments and are associated with mantle rocks. The first three fields present an intense hydrothermal activity and have active chimneys. A large number of small animals (mussels, shrimps, and crabs) and biofilms mainly composed of filamentous thermophilic prokaryotes can be found in association with these structures. The Saldanha field is characterized by a large methane anomaly in the water. On the seafloor, small holes with a reduced hydrothermal activity can be observed. At this field, the water temperature measured at the exit of the holes was only 3–4°C higher than the temperature measured a few centimeters from the holes. The Menez Hom mount (1780–1900 m) also features a large methane anomaly. Hydrothermal activity is still poorly understood at this field.

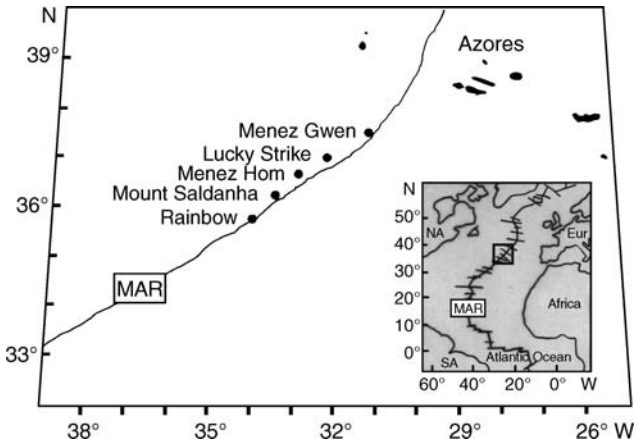

Figure 1. Map of the Mid-Atlantic Ridge and location of the sampling sites.

Sample Collection and Treatment. Seven water samples were collected using an insulated box previously filled with sterilized water. All samples were immediately processed on-board. One sample was collected in each visited site, except from Rainbow and Mount Saldanha for which two samples were collected in two independent dives. At Rainbow field, one of the samples was taken at

the vicinity of the black smokers (RB1) and the other one outside the active field (RB2). All samples were collected 3–5 m above the sea floor. Table 1 shows the coordinates and depths of each sample. The samples consisted of portions of 250–3000 mL of water that was filtered using sterile nitrocellulose filters (Millipore), with a pore size of 0.45 μm. The filters were subsequently placed on top of solid media. Two culture media were used: MYPss agar [0.7% (w/v) malt extract, 0.05% (w/v) yeast extract, 0.25% (w/v) peptone soytone, 3% (w/v) sea salts (Sigma), and 1.5% (w/v) agar] supplemented with 0.05% (w/v) chloramphenicol and MYPssS agar consisting of MYPss plus 0.605% (w/v) piperazine-*N*,*N*′-bis(ethanesulfonic acid) (PIPES) buffer (Sigma), 1% (w/v) sulfur, and 1.5% (w/v) agar, supplemented with 0.05% (w/v) chloramphenicol and with the pH adjusted to 7.5. MYPssS medium was autoclaved two times, on two consecutive days, at 100°C for 30 min. Depending on the origin of the sample, the petri dishes containing the filters and the culture media were incubated at 10°C for 45 days or at 22°C for 10 days. The incubation periods were set to obtain a good development of the yeast colonies, allowing therefore the detection of slow-growing species. Care was also taken to stop the incubation before yeast colonies were overgrown by filamentous fungi. For the Menez Gwen and Lucky Strike samples, the incubation temperature was 22°C since they were collected near hydrothermal vents. For the samples obtained at sites with low hydrothermal activity (Mount Saldanha and Menez Hom), the incubation temperature was 10°C. For sample RB1 (Rainbow field), incubations were performed at the two temperatures, and for sample RB2, incubations were performed at 10°C. All the colonies of pink yeasts were transferred and purified, whereas for the nonpigmented colonies, only a fraction of approximately 1% was retained for identification. After microscopic examinations were made to insure that no bacterial isolates had been selected, each purified culture was numbered under the MARY (Mid-Atlantic Ridge Yeast) acronym and stored at 4°C. All the cultures that were studied by sequence analysis were also cryopreserved in liquid nitrogen.

DNA Extraction. The protocol described by Gadanho *et al.* [4] was employed. Briefly, two loopfuls of MYP agar-grown cultures were suspended in 500 μL lysing buffer (50 mmol L^{-1} Tris, 250 mmol L^{-1} NaCl, 50 mmol L^{-1} EDTA, 0.3% w/v SDS, pH 8), and the equivalent to a volume of 200 μL of 425- to 600-μm glass beads (Sigma) was added. After vortexing for 3 min, the tubes were incubated for 1 h at 65°C. The suspensions were then centrifuged for 10 min. Finally, the collected supernatant was diluted 1:750, and 5 μL was directly used in the PCR. The remaining supernatant was immediately preserved at −20°C.

MSP-PCR Fingerprinting. The minisatellite primer M13 was used as previously described [2]. All PCR reactions were performed in 25-μL reaction volumes containing 1× PCR buffer (Amersham Biosciences), 2 mmol L^{-1} of each of the four dNTPs (Promega), 0.8 μmol L^{-1} of primer, 5 μL of the diluted supernatant containing the genomic DNA, and 1 U of *Taq* DNA polymerase (Amersham Biosciences). Amplification was performed in a Uno II Thermal Cycler (Biometra), consisting of an initial denaturation step at 95°C for 5 min, followed by 40 cycles of 45 s at 93°C, 60 s at 50°C, and 60 s at 72°C and a final extension step of 6 min at 72°C. A negative control in which DNA was replaced by sterile distilled water was also included. Amplified DNA fragments were separated by electrophoresis in 1.4% (w/v) agarose gel (Gibco BRL), in 0.5× Tris–borate–EDTA (TBE) buffer at 90 V for 3.5 h and stained with ethidium bromide. On each gel, a molecular size marker was used for reference (λ DNA cleaved with *Hin*dIII and ΦX174 DNA cleaved with *Hae*III, Amersham Biosciences).

DNA banding patterns were visualized under UV transillumination, and images were acquired using a Kodak Digital Science EDA 120 System and the Kodak Digital Science 1D Image Analysis Software. All fingerprints obtained were grouped by similarity using Gel Compar 4.1 (Applied-Maths) and the Pearson correlation coefficient. Visual confirmations of the MSP-PCR group fingerprints were performed, and minor adjustments were made. Finally, one representative strain of each group was chosen for sequence analysis of the D1/D2 domains of the 26S rDNA to obtain an identification down to the species level.

rDNA Sequencing. Total DNA was extracted using the method described above and amplified using rDNA

Table 1. Designations and locations of the sampling sites

Site	*Sample designation*	*Coordinates*	*Depth (m)*
Menez Gwen	MG	37°50.679′N, 31°31.138′W	825
Rainbow	RB1	36°13.764′N, 33°54.159′W	2295
Rainbow	RB2	36°13.773′N, 33°54.207′W	2316
Lucky Strike	LS	37°17.395′N, 32°16.643′W	1693
Mount Saldanha	MS1	36°35.251′N, 33°26.685′W	2116
Mount Saldanha	MS2	36°33.916′N, 33°25.887′W	2198
Menez Hom	MH	37°08.222′N, 32°26.609′W	1802

primers ITS5 (5′GGA AGT AAA AGT CGT AAC AAG G) and LR6 (5′CGC CAG TTC TGC TTA CC). Cycle sequencing of the 600- to 650-bp region D1/D2 at the 5′ end of the 26S rDNA domain employed forward primer NL1 (5′GCA TAT CAA TAA GCG GAG GAA AAG) and reverse primer NL4 (5′GGT CCG TGT TTC AAG ACG G). Sequences were obtained with an Amersham Pharmacia ALF express II automated sequencer using standard protocols.

Identification and Phylogenetic Analysis. For identification, the obtained sequences were compared with those of all known yeast species available at the GenBank database of the US National Center for Biotechnology Information (NCBI). For this task, the Basic Local Alignment Search Tool (BLAST) available at http://www.ncbi.nlm.nih.gov was used. For phylogenetic analyses, alignments were made with MegAlign (DNAStar) and visually corrected. To estimate phylogenetic relationships, the Bayesian Markov chain Monte Carlo method of phylogenetic inference (MCMC) [9] was applied as implemented in the computer program MrBayes [7]. Four incrementally heated simultaneous Monte Carlo Markov chains were run over 1,000,000 generations using the general time-reversible model (six rate classes) of DNA substitution, additionally assuming a portion of invariable sites with gamma-distributed substitution rates of the remaining sites (GTR + I + G), random starting trees, and default starting parameters of the DNA substitution model. Trees were sampled every 100 generations resulting in an overall sampling of 10,000 trees. From those trees that were sampled after the process had reached a stationary stage (burnin = 2000), a consensus tree was computed to obtain estimates for the *a posteriori* probabilities. This analysis was repeated three times, always using random starting trees and default starting values for the model parameters to test the reproducibility of the results.

Statistical Analysis. A chi-square analysis was performed to investigate the influence of the two culture media on yeast counts. The null hypothesis was that the media had no influence. Since in the analysis we had only two classes (MYPss and MYPssS), the Yates correction for continuity was used in the chi-square calculation [24].

Results

Yeast Isolation. Yeasts were found in all the surveyed sites except Lucky Strike. Yeast counts recorded for the two culture media and at the two incubation temperatures are depicted in Table 2. The number of cfu per liter ranged from 0 to 5940. The highest counts were obtained from the two samples taken at Rainbow field. At this site, depending on the sample, growth medium, and incubation temperature, the counts ranged from 4 to 5940 cfu/L for the nonpigmented yeasts and from 2 to 7 cfu/L for the pink yeasts. As shown in Table 2, higher counts were obtained from the sample taken outside the active field (RB2) than from the one taken within the hydrothermal field (RB1). The overall comparison of the total number of pigmented and nonpigmented yeasts clearly shows that the latter were detected in a much higher quantity. For the pink yeasts, the highest count was recorded in sample MS1 taken at Mount Saldanha and corresponded to 8 cfu/L.

To investigate the influence of the growth medium on detection and quantification of yeasts associated with deep-sea hydrothermal systems, a chi-square analysis was performed. The test allowed the rejection of the null hypothesis (i.e., the composition of the culture media had no effect) for pink and nonpigmented yeasts ($P < 0.001$). However, the influence of culture media was not the same for these two groups of yeasts. For the pink yeasts, the number of cfu was higher on MYPssS than on MYPss (Table 2), whereas for the nonpigmented yeasts, the opposite effect was observed, except for sample RB2. Sample RB1 was tested at two different incubation temperatures (10 and 22°C). As shown in Table 2, a higher number of nonpigmented colonies were detected at 22°C, whereas for pigmented yeasts, more cfu were

Table 2. Yeast counts (cfu/L) obtained using different culture media and different incubation temperatures (ND, not determined)

	Nonpigmented yeasts				*Pink yeasts*			
	MYPss		*MYPssS*		*MYPss*		*MYPssS*	
Site	*22°C*	*10°C*	*22°C*	*10°C*	*22°C*	*10°C*	*22°C*	*10°C*
Menez Gwen (MG)	12	ND	12	ND	0	ND	0	ND
Rainbow (RB1)	200	46	1200	4	2	4	3	6
Rainbow (RB2)	ND	4752	ND	5940	ND	3	ND	7
Lucky Strike (LS)	0	ND	0	ND	0	ND	0	ND
Mount Saldanha (MS1)	ND	204	ND	135	ND	1	ND	8
Mount Saldanha (MS2)	ND	40	ND	20	ND	0	ND	2
Menez Hom (MH)	ND	384	ND	58	ND	2	ND	3

detected at 10°C. However, given that the counts for pink yeasts were low and this comparative study was only carried out for Rainbow (RB1) samples, the significance of these results has to be analyzed with care.

For the pink yeasts, 95 cfu were counted and purified. For the nonpigmented yeasts, because of the high counts that were recorded, approximately 1% of the colonies were purified. Therefore, 97 nonpigmented colonies were selected based on different colony morphology and color tone. An additional criterion for the selection of nonpigmented yeasts was that they should include isolates obtained from the different temperatures of incubation and the two culture media tested, as well as from the four MAR sites where yeasts were found. Thus, a total of 192 isolates (97 nonpigmented and 95 pink colored) were purified for further analysis.

MSP-PCR Fingerprinting. To assess the diversity of the isolates, MSP-PCR fingerprinting employing the minisatellite primer M13 was used. With this approach, MSP-PCR similarity classes were formed and two dendrograms were constructed, one grouping the PCR profiles obtained for the nonpigmented yeasts (Fig. 2) and the other comprising the profiles obtained for the pink isolates (Fig. 3). With this strategy, a total of 12 MSP-PCR classes (classes 1–12) were obtained for the nonpigmented yeasts and 11 classes (classes 13–23) were defined for the pink yeasts. The minor fingerprint variability found within some MSP-PCR classes was regarded as intraspecific heterogeneity or as variability associated with the method. For the nonpigmented yeasts (Fig. 2), the majority of the isolates studied (67%) belonged to MSP-PCR classes 3 (31% of the isolates), 7 (19%), and 10 (17%). Three classes were composed of a single isolate (classes 2, 8, and 12), and two classes had two isolates (classes 9 and 11). Almost half of the pink isolates shown in Fig. 3 were grouped in classes 21 and 22 with 22 and 27% of the total of pink isolates, respectively. The remaining pink yeasts were distributed in nine classes. All those classes except one (class 18) consisted of more than one isolate.

26S rDNA Sequence Analysis. For species identification, one isolate of each MSP-PCR class was selected for sequence analysis of the D1/D2 domains of the 26S rDNA. A total of 19 phylotypes were found among the yeasts isolated from MAR hydrothermal systems. Eleven phylotypes could be assigned to known yeast species. In total, 12 phylotypes belonged to the Ascomycota and 7 to the Basidiomycota (Fig. 4). Among the nonpigmented yeasts (Fig. 4A), all sequences, except the one corresponding to MSP-PCR class 1 (*Trichosporon dermatis*), were from ascomycetes. The other species that were identified with this approach (100% similarity with reference sequences) were *Candida atlantica* (MSP-PCR class 6), *C. atmosphaerica* (class 10), *C. lodderae* (class 3), *C. parapsilosis* (class 11), *Exophiala dermatitidis* (class 8), and *Pichia guilliermondii* (class 7).

All the pigmented yeasts found at MAR hydrothermal systems were affiliated with the Basidiomycota (Fig. 4B). The species for which a conclusive identification was obtained (100% similarity with reference sequences) were *Rhodosporidium diobovatum* (MSP-PCR classes 19 and 20), *R. sphaerocarpum* (class 23), *R. toruloides* (classes 13 and 14), and *Rhodotorula mucilaginosa* (classes 17, 21, and 22) (Fig. 4B). Except for *R. sphaerocarpum*, more than one MSP-PCR class was found for each species. All these species belong to the order Sporidiobolales of the class Urediniomycetes.

Discussion

New Phylotypes. Eight new phylotypes were obtained. Therefore, it is likely that they represent undescribed species. Five new sequences were detected among the ascomycetous yeasts, and the phylogenetic analysis of these sequences is shown in Fig. 4A. MARY 148 has as closest neighbors *Candida* sp. CBS 7880 (100% similarity), *Candida insectorum* and *Pichia mexicana* (both with 19 mismatches), and *Pichia scolyti* (20 mismatches). The closest relative of MARY 179 is *C. membranaefaciens* (6 mismatches). The remaining three new sequences (MARY 089, MARY 092, and MARY 101) are allocated in the *C. atlantica–C. atmosphaerica* clade. The strain MARY 092 differs from *C. atlantica* by 8 mismatches, and MARY 089 and MARY 101 have 21 and 14 mismatches toward *C. atmosphaerica*, respectively.

For the basidiomycetous yeasts, three phylotypes could not be assigned to any of the known yeast species. The sequence obtained for MARY 160 (MSP-PCR class 18) is closely related to *Rh. mucilaginosa* but shows three mismatches (Fig. 4B). Therefore, its relationship with *Rh. mucilaginosa* needs further study. The sequences obtained for MARY 063 (MSP-PCR class 15) and MARY 297 (MSP-PCR class 16) differ only by one nucleotide, and so these strains are provisionally considered as conspecific. The two sequences were assigned to the *Naohidea–Rhodotorula minuta* clade of the Urediniomycetes, and their closest relative was *Rhodotorula lamellibrachii* (with 17 and 18 mismatches, respectively).

In conclusion, a considerable fraction (33%) of the 18 yeast taxa that were found in MAR hydrothermal systems seems to represent undescribed species based on molecular sequence comparisons. It is therefore possible that the new phylotypes may represent autochthonous species, but a more detailed picture on this issue will only emerge after more data are gathered.

Influence of Culture Media. Similarly to what has been recognized in diversity studies of prokaryotes, it is

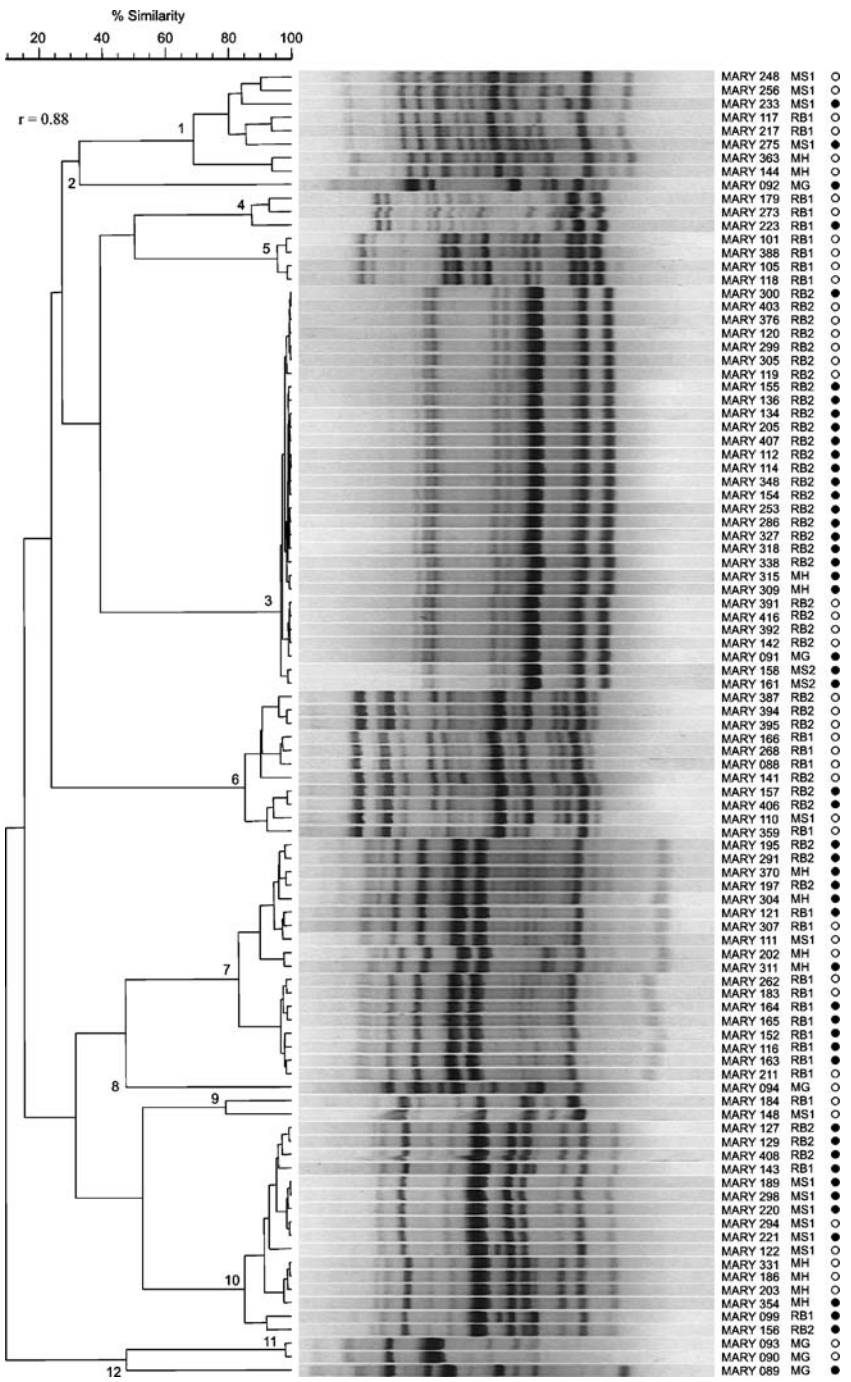

Figure 2. MSP-PCR dendrogram and fingerprints of nonpigmented yeasts obtained with primer M13 (Pearson coefficient, Unweighted Pair Group Method with Arithmetic mean clustering, r = cophenetic correlation coefficient). Numbers in the *branches* indicate the similarity clusters. The sample from which each isolate was obtained is indicated after the isolate number. *Empty circles* indicate isolates obtained on MYPss medium, and *black circles* indicate isolates obtained on MYPssS medium.

likely that the artificial growth conditions used in conventional yeast isolation do not allow the correct assessment of yeast diversity. An argument for the existence of such a bias was obtained in the present study since it was observed that the use of MYPss or MYPssS affected significantly the yeast counts. Higher counts of nonpigmented yeast were obtained using MYPss, whereas for pink yeasts, higher counts were obtained using MYPssS. Moreover, some of the MSP-PCR classes of pink yeasts were obtained only on MYPssS. This was the case of classes 14, 16, 18, and 20 whose members were isolated exclusively on MYPssS. Among the nonpigmented yeasts, classes 2 and 12 (both with single isolates) were MYPssS-specific, and classes 5, 8 (single isolate), 9, and 11 were MYPss-specific. It should be noted that for the nonpigmented yeasts, the observed correlations can be affected by the fact that only a fraction of the nonpigmented yeasts detected was studied in

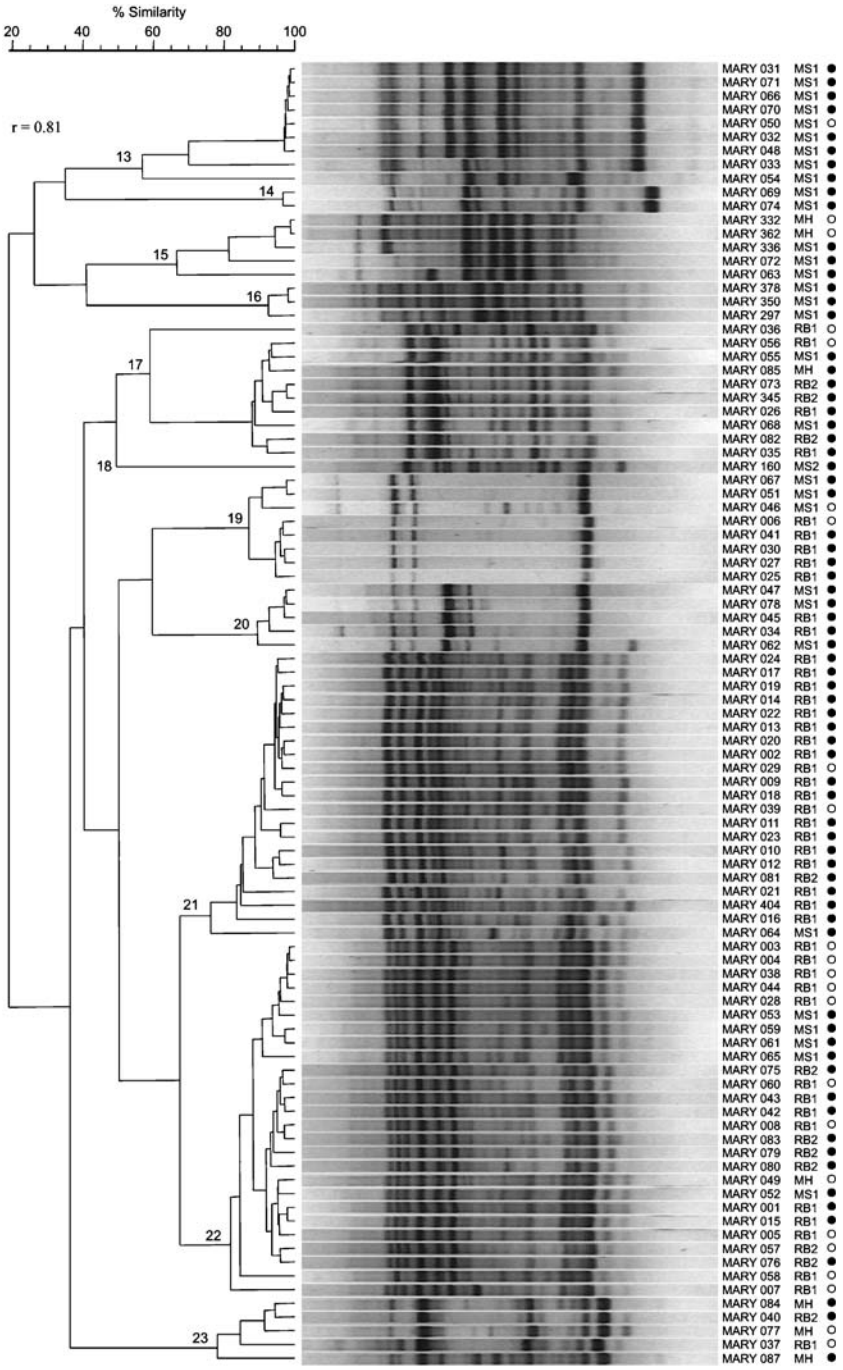

Figure 3. MSP-PCR dendrogram and fingerprints of pigmented yeasts obtained generated with primer M13. See legend of Fig. 2 for details.

contrast with the group of pink yeasts for which all isolates were studied.

Structure of the Yeast Community. As shown in Table 2, the counts for nonpigmented yeasts, which are represented mainly by ascomycetous species, were much higher than those obtained for pink yeasts, a group composed solely of basidiomycetous species. In this study, the total number of cfu per liter of pigmented yeasts corresponded to 0.3% of the total number of yeasts found. This disproportion was not observed in studies of other marine systems (for a review, see [5]) and may be due to the unique conditions of hydrothermal vents. These environments are characterized by a considerable animal and microbial diversity [1, 21], and it is conceivable that they are rich in organic compounds utilizable by yeasts. Conversely, in open sea or deep-sea benthic environments, nutrients are probably scarcer, a

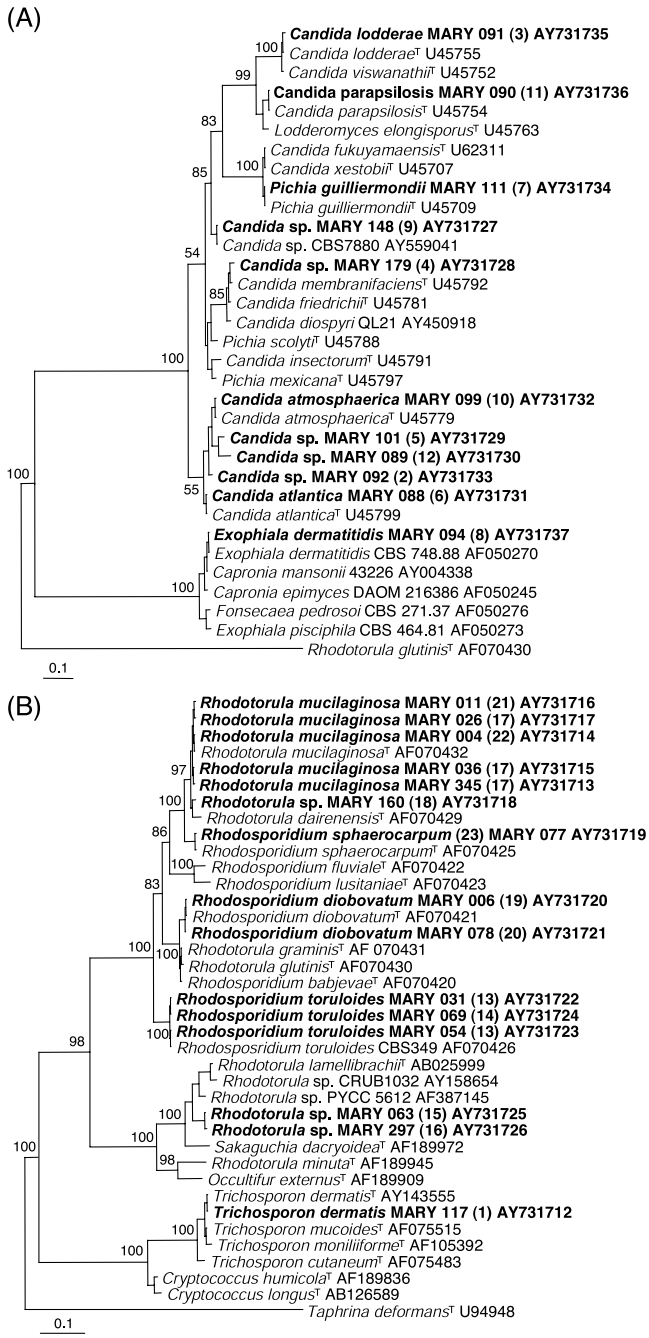

Figure 4. Phylogenetic trees including the phylotypes obtained in this study and reference sequences retrieved from the GenBank database. The phylogenetic relationships were inferred using the Bayesian Markov chain Monte Carlo analysis of alignments of the D1/D2 domains of the 26S rDNA. Numbers on the *branches* are estimates for *a posteriori* probabilities, i.e., probabilities that the respective groups are monophyletic given the alignment. Names in *boldface* correspond to the sequences determined in this study, and numbers between *brackets* refer to the MSP-PCR classes of Figs. 2 and 3 (^{T} = type strain). (A) Comparison of sequences of ascomycetous yeasts rooted with *Rhodotorula glutinis*; (B) comparison of sequences of basidiomycetous yeasts rooted with *Taphrina deformans*.

situation that seems to be less detrimental for pigmented yeasts (basidiomycetous yeasts) than for the nonpigmented ones (mostly ascomycetous) [14]. For example, in yeast isolations from deep-sea sediments in the northwest Pacific Ocean, it was found that the proportion of pigmented yeasts was 10.6% in sediments collected at depths less than 2000 m, whereas at depths ranging from 2000 to 6500 m, the proportion increased to 64% [14]. Besides nutrient availability, another factor that can influence the distribution of these two groups of yeasts is their possible unequal tolerance to increased values of hydrostatic pressure. In another study of yeast occurrence and distribution in the water column of coastal waters (0–700 m) off the south of Portugal, the proportion of pigmented yeasts was approximately 60% [4]. Recent studies involving the enrichment of estuarine yeast populations prior to their molecular detection have indicated that the ascomycetous yeasts dominate the first 3 days of incubation, whereas normally basidiomycetous yeasts were only detectable a few days later [3]. Thus, it is possible that the combined effect of much denser populations of nonpigmented yeasts and of their faster growth rate in the first days of incubation has hindered the development of pigmented colonies. In the present study, the low proportion of pigmented yeasts seems to be a distinctive feature of the yeast community associated with the surveyed hydrothermal systems.

Some of the yeasts belong to phylogenetic groups that include species reported from other marine environments. Among the ascomycetous yeasts, *C. atlantica* of the *C. atlantica–C. atmosphaerica* clade was found in shrimp eggs and in coastal waters in the south of Portugal [4]. This species has been also found in our study together with two phylogenetically related but undescribed yeasts (Fig. 4A). With respect to basidiomycetous yeasts, the taxon represented by strains MARY 063 and MARY 297 in Fig. 4B belongs to a clade that includes *Rh. lamellibrachii*, a species found in a tubeworm collected in Sagami Bay, Japan [14]. Other species that might be adapted to marine environments, including the hydrothermal systems, are *P. guilliermondii*, which was found in the present study and in estuarine waters [3], and *C. lodderae*, previously reported from a shrimp in the Gulf of Mexico and one of the most abundant yeasts in our study. Table 3 depicts the occurrence of selected yeast species identified using molecular methods, in different types of aquatic environments. Interestingly, *Debaryomyces hansenii*, a common yeast in seawater, was not detected in our study. With respect to pigmented yeasts of the genus *Rhodosporidium*, available data suggest that species like *R. babjevae* and *R. kratochvilovae* might be allochthonous to the marine habitat, whereas *R. diobovatum* and *R. sphaerocarpum* are good candidates for autochthonous yeasts. In this context, *R. toruloides* might represent a special case in the genus

Table 3. Presence (+) or absence (−) of selected yeast species in different types of aquatic environments (ND, not determined)

Species	*Hydrothermal systems, MAR (this study)*	*Deep-sea, Pacific Ocean, [14]*	*Coastal, south of Portugal [4]*	*Estuary, Tagus river, Portugal [3]*	*Oligotrophic lakes, Patagonia, Argentina [11]*
Nonpigmented					
C. atlantica	+	ND	+	−	−
C. atmosphaerica	+	ND	−	−	−
C. lodderae	+	ND	−	−	−
C. parapsilosis	+	ND	+	−	−
D. hansenii	−	+	+	+	−
P. guilliermondii	+	ND	−	+	−
Pigmented					
R. babjevae	−	−	+	−	+
R. diobovatum	+	+	+	−	−
R. kratochvilovae	−	−	+	−	+
R. sphaerocarpum	+	+	+	−	−
R. toruloides	+	−	−	−	−
Rh. mucilaginosa	+	+	+	+	+
Rh. minuta	−	+	+	−	+
S. dacryoides	−	−	+	−	−

C.: *Candida*; *D.*: *Debaryomyces*; *P.*: *Pichia*; *R.*: *Rhodosporidium*; *Rh.*: *Rhodotorula*; *S.*: *Sakaguchia*.

Rhodosporidium because besides having been found in the present study, it shows a tendency to be present in aquatic habitats that feature strong environmental stresses caused by toxic compounds or low pH (Gadanho and Sampaio, unpublished). In the genus *Rhodotorula*, Rh. mucilaginosa shows a remarkable ubiquity (Table 3) and is found in different types of aquatic environments [3, 4, 11], including deep-sea habitats [14]. Among the pink yeasts isolated in this study, 60% of the isolates belong to *Rh. mucilaginosa*. Another species that was consistently reported in the past from marine environments is *Rh. glutinis* [5]. The ecology of this species needs a reassessment as identifications based on growth responses often fail to differentiate *Rh. glutinis* from *R. babjevae* and several other species [2].

The hydrothermal systems surveyed in our study can be classified in two groups according to their high (Lucky Strike, Menez Gwen, and Rainbow) or low (Menez Hom and Mount Saldanha) geochemical activity. Some yeasts like the undescribed *Candida* species of MSP-PCR classes 2, 5, and 12 were only found at the more active sites, whereas *R. toruloides* and the two undescribed pigmented species were found exclusively in the less active sites. Further studies are needed to elucidate if these findings result from different ecological niches that these yeast species occupy at MAR hydrothermal fields.

Acknowledgments

The authors wish to thank the scientific members of the SEAHMA-I mission, the crew of L'Atalante, and the ROV operating personnel for their help during this study. Part of this work was supported by project SEAHMA—Seafloor and subseafloor hydrothermal modeling in the Azores Sea, PDCTM/C/MAR/15281/99. M. Gadanho was supported by a grant SFRH/BD/1170/2000.

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