

Morphological Response of the Halophilic Fungal Genus *Wallemia* to High Salinity[∇]

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The basidiomycetous genus *Wallemia* is an active inhabitant of hypersaline environments, and it has recently been described as comprising three halophilic and xerophilic species: *Wallemia ichthyophaga*, *Wallemia muriae*, and *Wallemia sebi*. Considering the important protective role the fungal cell wall has under fluctuating physicochemical environments, this study was focused on cell morphology changes, with particular emphasis on the structure of the cell wall, when these fungi were grown in media with low and high salinities. We compared the influence of salinity on the morphological characteristics of *Wallemia* spp. by light, transmission, and focused-ion-beam/scanning electron microscopy. *W. ichthyophaga* was the only species of this genus that was metabolically active at saturated NaCl concentrations. *W. ichthyophaga* grew in multicellular clumps and adapted to the high salinity with a significant increase in cell wall thickness. The other two species, *W. muriae* and *W. sebi*, also demonstrated adaptive responses to the high NaCl concentration, showing in particular an increased size of mycelial pellets at the high salinities, with an increase in cell wall thickness that was less pronounced. The comparison of all three of the *Wallemia* spp. supports previous findings relating to the extremely halophilic character of the phylogenetically distant *W. ichthyophaga* and demonstrates that, through morphological adaptations, the eukaryotic *Wallemia* spp. are representative of eukaryotic organisms that have successfully adapted to life in extremely saline environments.

Hypersaline habitats had long been considered to be populated almost exclusively by prokaryotic organisms and the research on hypersaline environments had consequently been monopolized by bacteriologists. In 2000, the first reports appeared showing that fungi are active inhabitants of solar salt-erns (20). Until then, fungi able to survive in environments with a low amount of biologically available water (low water activity [a_w]) were only known as contaminants of foods preserved with high concentrations of salt or sugar. Since their first discovery in salterns, many new species have been discovered in natural hypersaline environments around the world, including some species that were previously known only as food-borne contaminants. Due to these discoveries, fungi are now recognized as an integral part of indigenous halophilic microbial communities since they can grow and adjust across the whole salinity range, from freshwater to almost saturated NaCl solutions (49). Most fungi differ from the majority of halophilic prokaryotes (16): they tend to be extremely halotolerant rather than halophilic and do not require salt to remain viable, with the exception of *Wallemia* spp.

The order *Wallemiales* (Wallemiomycetes, Basidiomycota) was only recently introduced to define the single genus *Wallemia*, a phylogenetic maverick in the Basidiomycota (49). Until 2005, this genus contained only the species *W. sebi*. However, taxonomic analyses of isolates from sweet, salty, and dried foods (41) and from hypersaline evaporation ponds in the Mediterranean Sea, the Caribbean, and the Dead Sea (45, 49) have resolved this genus into three species: *W. ichthyophaga*,

W. muriae, and *W. sebi*. The first two of these three *Wallemia* spp. require additional solutes in the growth media, and *W. ichthyophaga* is the most halophilic eukaryote described to date, since it cannot grow without the addition of 9% NaCl (wt/vol), and it still shows growth at a_w of 0.77, equivalent to 30% NaCl (wt/vol) (49).

The survival, and especially the growth, of microorganisms in highly saline environments requires numerous adaptations (6, 18, 21, 34). The dominant representatives and the most thoroughly investigated halophilic fungi in hypersaline waters of the salterns are the black yeasts, and particularly the model organism *Hortaea werneckii* (20). An important level of adaptation of the black yeasts to high salinity is seen in their extremophilic ecotype, which is characterized by a special meristematic morphology and changes in cell wall structure and pigmentation (27). Other fungal osmoadaptations include the accumulation of osmolytes (27, 28, 40), the extrusion of sodium (5), modification of the plasma membrane (44) and the cell wall, and even changes in fungal colony morphology (27).

The fungal cell wall is the first line of defense against environmental stress; therefore, adaptation at the cell wall level is expected to have one of the most important roles for successful growth at a low a_w (24, 32). The cell wall is essential for maintaining the osmotic homeostasis of cells, since it protects them against mechanical damage as well as high concentrations of salts (7). The central fibrillar glycan network of the cell wall is embedded in highly flexible amorphous cement, which allows considerable stretching with changing osmotic pressure (14, 29). Its balance between a rigid and a dynamic structure influences the shape of cells (14) and enables growth and hyphal branching (11).

Since the species within the genus *Wallemia* have been recognized only recently (49), little is known about their mecha-

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nisms of adaptation to high salinity. To investigate the effects of low and high NaCl concentrations on cell morphology, with particular emphasis on cell wall ultrastructure, we compared *W. ichthyophaga*, the most halophilic fungal species known thus far, with the related xerophilic *W. muriae* and *W. sebi*. Micrographs were prepared by using light, transmission, and scanning electron microscopy. The results reveal how this eukaryotic genus uses adaptations at the cell wall level for thriving in extremely saline environments.

MATERIALS AND METHODS

Organisms and culture conditions. The fungi under study were maintained in the Culture Collection of Extremophilic Fungi (EXF) of the Department of Biology, Biotechnical Faculty, University of Ljubljana (Ljubljana, Slovenia) and in the Centraalbureau voor Schimmelcultures (CBS; Utrecht, The Netherlands). *Wallemia ichthyophaga* EXF-994 (CBS 113033) and *Wallemia muriae* EXF-951 (CBS 116628) were isolated from hypersaline waters of the Sečovlje Adriatic solar saltern in Slovenia. *Wallemia sebi* EXF-958 (CBS 818.96) was isolated from sunflower seeds in Sweden (49).

Growth conditions and determination of growth characteristics. Cultures of *W. ichthyophaga* were maintained on solid malt extract medium (2% malt extract [19]) with 10% NaCl, and those of *W. muriae* and *W. sebi* were maintained on malt extract, yeast extract, and 50% glucose agar (MY50G; 2% malt extract, 0.5% yeast extract [39]); all were stored at 4°C.

These fungi were cultured in liquid and on solid yeast nitrogen base (YNB) medium [1.7 g of YNB, 5 g of (NH₄)₂SO₄ per liter, 0.8 g of complete supplement mixture per liter, 20 g of glucose per liter, 20 g of agar per liter for solid medium (pH 7.0)]. For determination of the growth across the NaCl concentration range, the a_w of the media was adjusted with NaCl [0 to 30% (wt/vol)]. The growth media were autoclaved at 121°C for 15 min. Cultures in liquid media were incubated in the dark at 28°C with constant shaking at 180 rpm. Cultures on solid YNB medium were incubated in the dark at 24°C.

For each of the *Wallemia* spp., growth was monitored at one low and one high salinity (*W. ichthyophaga* at 15 and 25% [wt/vol] NaCl; *W. muriae* and *W. sebi* at 5 and 20% [wt/vol] NaCl). Preculturing on the YNB medium of the same salinity as in the experiment was used to adapt the fungi to the media and growth conditions used. The cultures for the experiments were inoculated from precultures in the exponential phase of growth (inoculum was 1% of the final culture volume). The growth of the fungal strains in the liquid YNB medium was determined from samples every 2 days. The dry biomass weight was used, since the formation of densely interwoven mycelial masses referred to as cell pellets did not allow reliable measures of the optical density. The suspension of the pellets was filtered through nitrocellulose filter (pore size, 1.2 μm) and dried at 100°C to a constant weight. The growth curves were constructed from two independent experiments, each carried out in duplicate, and used to determine the final fungal biomass yield. Growth rates and generation times were calculated from the doubling times obtained from the growth curves during the exponential growth phase.

Sample preparation for light microscopy. Cells from cultures grown to the mid-exponential growth phase were analyzed with an Olympus BX51 light microscope equipped with an Olympus DP12 digital camera. The cell sizes and sizes of hyphal compartment and multicellular clumps were measured in DP-Soft 3.2 (Olympus).

Sample preparation for transmission electron microscopy (TEM). Cells from cultures grown to mid-exponential growth phase were filtered through nitrocellulose filter (pore size, 1.2 μm) and fixed in 2.5% glutaraldehyde and 4% paraformaldehyde buffered in 0.1 M sodium phosphate buffer (pH 7.2) for 2 h at room temperature. NaCl was added into the fixative to the same osmolality as in the growth media. After fixing, the cells were rinsed three times with 0.1 M sodium phosphate buffer with descending concentrations of NaCl. This optimization of the fixation procedure was necessary since the standard fixation without NaCl caused osmotic shock and a collapse of the cell structure. Due to their small size, the samples were embedded in 3% agarose, and cut into small blocks of about 1 by 1 by 5 mm. Agarose embedding prevented cell loss during the processing and also allowed better penetration of chemicals into the fungal cells. Postfixing was performed in 1% OsO₄ in distilled water with a drop of 0.1 M sodium phosphate buffer, for 24 h at 4°C. After three washes in distilled water, the samples were dehydrated through a graded series of ethanol solutions (vol/vol): 30% (10 min), 50% (twice for 10 min each time), 70% (twice for 10 min each time), 80% (twice for 10 min each time), 90% (twice for 10 min each time),

absolute ethanol (three times for 10 min each time). Agar 100 resin (Agar Scientific) was used for embedding. Ultrathin sections (70 to 90 nm) were cut with a diamond knife and contrasted with uranyl acetate and lead citrate. The sections were analyzed with a Philips CM 100 transmission electron microscope (80 kV, with a Gatan Bioscan Camera 792 digital camera) using Digital Micrograph 3.3.1.

Sample preparation for standard and focused-ion-beam/scanning electron microscopy (FIB/SEM). A focused-ion-beam scanning electron microscope is a system with both electron and ion beam columns, allowing the same sample to be investigated using either of the beams. A focused beam of ions, usually gallium, is used to image the sample in the chamber and to section the surface to expose the interior.

Cells from cultures grown to mid-exponential growth phase were filtered through a polycarbonate filter placed into a polypropylene filter holder, using a plastic syringe (Agar Scientific). All preparation steps, including the fixing and dehydration, were similar for TEM and for both standard and focused-ion-beam SEM. For standard and focused-ion-beam scanning electron microscopy, the samples were not embedded but were instead dried in 1,1,1,3,3,3-hexamethyl-disilazane for 45 min at room temperature. After drying, the cells were mounted on aluminum stubs and coated with gold by magnetron sputtering (Bal-Tec SCD 050 sputter coater). The samples were analyzed with an FEI Strata DB 235 M scanning electron microscope or a Sirion 400 NC focused-ion-beam/scanning electron microscope (17).

Statistical analyses. The number of samples analyzed is shown in legend to Fig. 3. The differences among the medians of the cell and cluster sizes, as well as the hyphal compartment lengths of all three of the *Wallemia* spp. at the low and the high salinities, were compared by using the Mann-Whitney U test. The differences between the medians of the cell wall thicknesses were compared by Kruskal-Wallis test, which determined whether the median of the cell wall thicknesses of *W. ichthyophaga* differed significantly from those of *W. muriae* and *W. sebi*. All calculations were done by using STAT-GRAPHICS Plus 4.0 statistics software for Windows. Statistical differences between two salinities were categorized into three groups (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

RESULTS

Growth of *Wallemia* spp. at various salinities. *W. ichthyophaga*, *W. muriae*, and *W. sebi* were grown in liquid YNB media supplemented with a broad range of NaCl concentrations. Growth tests showed that *W. ichthyophaga* grew from 9 to 30% (wt/vol) NaCl, whereas the *W. muriae* NaCl growth range extended from 4 to 25% (wt/vol) NaCl. *W. sebi* grew at up to 27% NaCl and was the only one of the tested species that also grew on media without NaCl (Table 1).

According to their growth across the NaCl concentration range, we selected two NaCl concentrations for each of the species to compare the influence of salinity on the morphological characteristics of *Wallemia* spp. Therefore, all further investigations were carried out at a low NaCl concentration (15% NaCl for *W. ichthyophaga* and 5% NaCl for *W. muriae* and *W. sebi*) and a high NaCl concentration (25% NaCl for *W. ichthyophaga* and 20% NaCl for *W. muriae* and *W. sebi*). The growth parameters, including the specific growth rate, the final fungal biomass yield, and the duration of lag phase were obtained from the growth curves of each of these species at each of the above-mentioned salinities, and are presented in Table 1.

In the media with the high NaCl concentrations, the duration of the lag phase prolonged for 2 days in all three *Wallemia* spp. Furthermore, the growth rates and the final biomass yields of all of the three species were also reduced. At the high salinities, *W. ichthyophaga* reached 87% of its final biomass yield produced at the low salinity, whereas *W. muriae* and *W. sebi* reached only 54 and 77%, respectively, of the final biomass they produced at the low salinity.

TABLE 1. Growth parameters of *Wallemia* spp. at low and high salinities^a

Organism	Growth range (% NaCl)	Specific growth rate (g g ⁻¹ h ⁻¹)		Final biomass yield (mg/100 ml of medium)		Duration of lag phase (days)	
		LS	HS	LS	HS	LS	HS
<i>W. ichthyophaga</i>	9–30	0.123	0.083	514.3	449.2	5	7
<i>W. muriae</i>	4–25	0.078	0.045	66.5	35.8	2	4
<i>W. sebi</i>	0–27	0.164	0.057	83.8	64.1	1	3

^a LS, low salinity: 5% NaCl for *W. muriae* and *W. sebi* and 15% NaCl for *W. ichthyophaga*. HS, high salinity: 20% NaCl for *W. muriae* and *W. sebi* and 25% NaCl for *W. ichthyophaga*.

Morphology and cell wall ultrastructural changes in *W. muriae* and *W. sebi*. Since similar morphological characteristics were seen for the more closely related species of *W. muriae* and *W. sebi*, these two species are presented together. On solid YNB medium at 5 and 20% NaCl, the colonies of *W. muriae* (Fig. 1a and b; Table 2) and *W. sebi* (Fig. 1g and h) differed in

size, color, and the extent of the area that was in direct contact with the medium.

In submerged culture, *W. muriae* and *W. sebi* grew in filamentous form at both low and high salinities (Fig. 1c, d, i, and j). Filamentous hyphae branched and intertwined into compact mycelial pellets, densely interwoven mycelial masses. At the

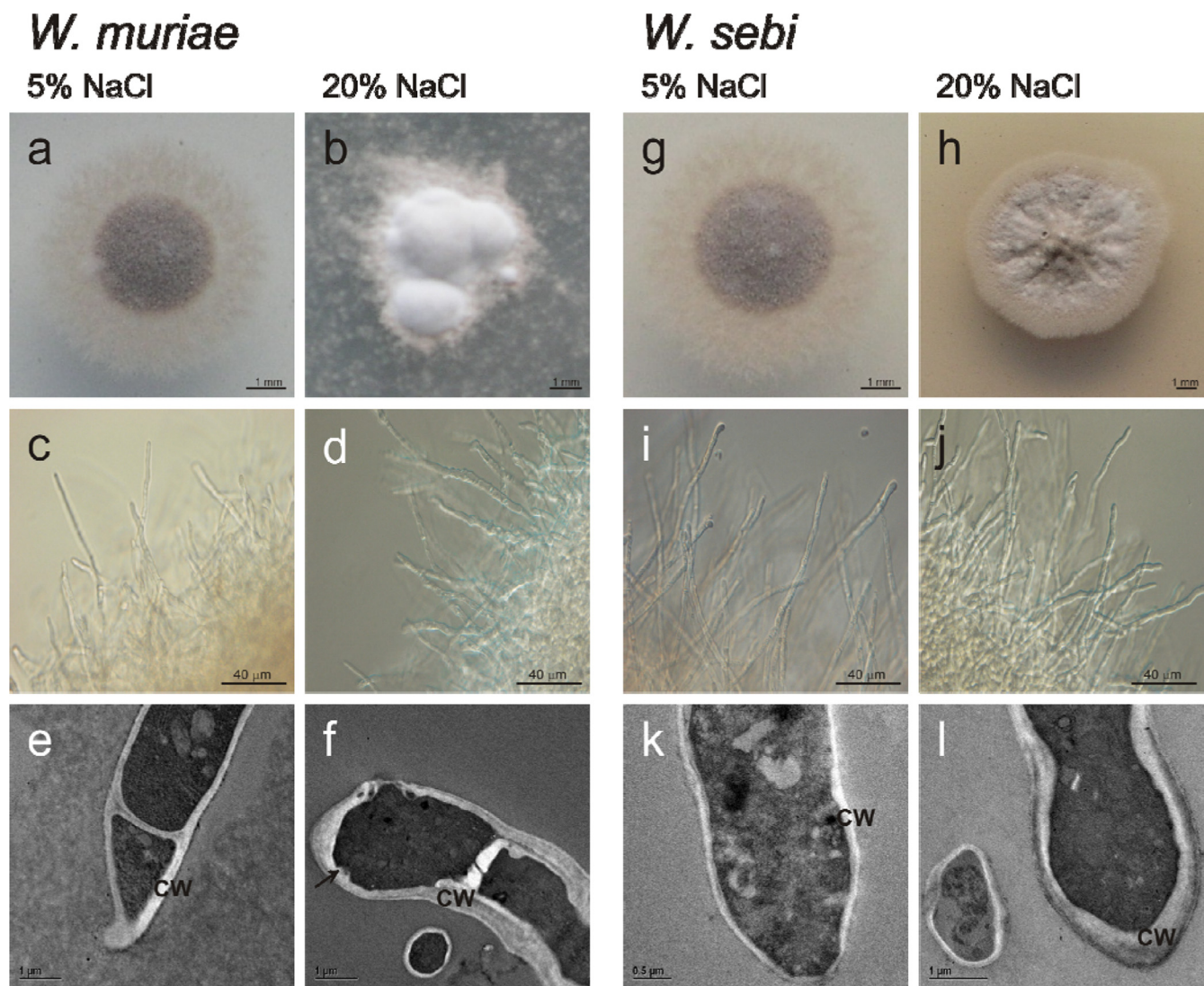


FIG. 1. NaCl effects on morphology of *W. muriae* and *W. sebi*. *W. muriae* (a, b, c, d, e, and f) and *W. sebi* (g, h, i, j, k, and l), showing colonies at 5% (a and g) and 20% (b and h) NaCl, hyphal morphology at 5% (c and i) and 20% (d and j) NaCl, and TEM micrographs at 5% (e and k) and 20% (f and l) NaCl. CW, cell wall; arrow, cell wall indentation.

TABLE 2. Morphological characteristics of *Walleimia* spp.

Organism	% NaCl in growth medium	Cell type or morphology	Colony morphology on solid medium	Presence or absence of ^a :	
				Cell wall indentations	EPS
<i>W. ichthyophaga</i>	15	Multicellular clumps	Light brown to yellowish/olive green; punctiform; heaped; soft; spreading deeply into the agar; yellow reverse (see Fig. 4a)	+	+++
	25	Multicellular clumps	White, yellowish to light green; heaped; soft; did not spread deeply into the agar; reduced area in contact with agar; light yellow reverse (see Fig. 4b)	++	++
<i>W. muriae</i>	5	Hyphae	Brown; round shape with light margin; powdery (high no. of spores), dry; spreading deeply into the agar; brown reverse (see Fig. 1a)	-	+
	20	Hyphae	Light brown to white; when white also dusty (high no. of spores); variable shape with light beige shaggy margin; heaped, powdery, dry; spreading into the agar; reduced area in contact with agar, pronounced aerial hyphae; brown reverse (see Fig. 1b)	+	+
<i>W. sebi</i>	5	Hyphae	Light brown, with the center being lighter, with light tan hyphae; round shape with light shaggy marginal surface; powdery (high no. of spores), dry; spreading deeply into the agar; brown reverse (see Fig. 1g)	-	+
	20	Hyphae	Rust brown with light margin, off white in the center; powdery, dry; spreading into the agar, not deeply; reduced area in contact with agar, brown reverse (see Fig. 1h)	+	+

^a -, Not present; +, present; ++, pronounced; +++, abundant.

low NaCl concentration, these pellets were darker and round shaped, whereas at the high salinity, they were light brown and variable in shape (Fig. 2a and b). Hyphal tips at the outer part of mycelial pellets were shorter at the high salinity (Fig. 2a and b). Furthermore, the morphology of the mycelial pellets at the low and high salinities differed significantly in size (*W. muriae*, $P < 0.01$; *W. sebi*, $P < 0.01$; Fig. 3a). At the high salinities, the mycelial pellets of *W. muriae* and *W. sebi* were four- and three-fold larger, respectively (Fig. 3a). The high NaCl concentration resulted in a significant increase in hyphal diameter for both *W. muriae* and *W. sebi* and a decrease in the hyphal compartment length, which was significant for both species (Fig. 3b and c). Also, the hyphal branching was more pronounced, compared to the low salinity. These cells did not aggregate into multicellular clumps at any of the salinities tested. Indeed, scanning electron micrographs of *W. muriae* and *W. sebi* mycelial pellets

(Fig. 2c) showed relatively smooth and regular hyphal surfaces at both of the salinities tested.

The analyses of the transmission electron micrographs of *W. muriae* and *W. sebi* hyphae at the low salinity (5% NaCl) showed less variability in the hyphal diameters (Fig. 3b) and pronounced pigmentation of the cell wall. The cell wall thickness was ca. 0.2 μm . At the high salinity, there was a minor, and not significant, increase in cell wall thickness in both of these species (Fig. 3d). In addition, with both *W. muriae* and *W. sebi*, cell wall indentations appeared, and the intracellular vacuolation changed. In contrast to larger vacuoles in *W. muriae* and *W. sebi* grown at the low salinity, at the high salinity smaller but numerous vacuoles were observed. Independent of the NaCl concentrations in the growth media, the outer cell wall layers of both species were covered with a small amount of unpronounced fibrous extracellular polymeric substances (EPS) (Table 2; see Fig. 6b and c).

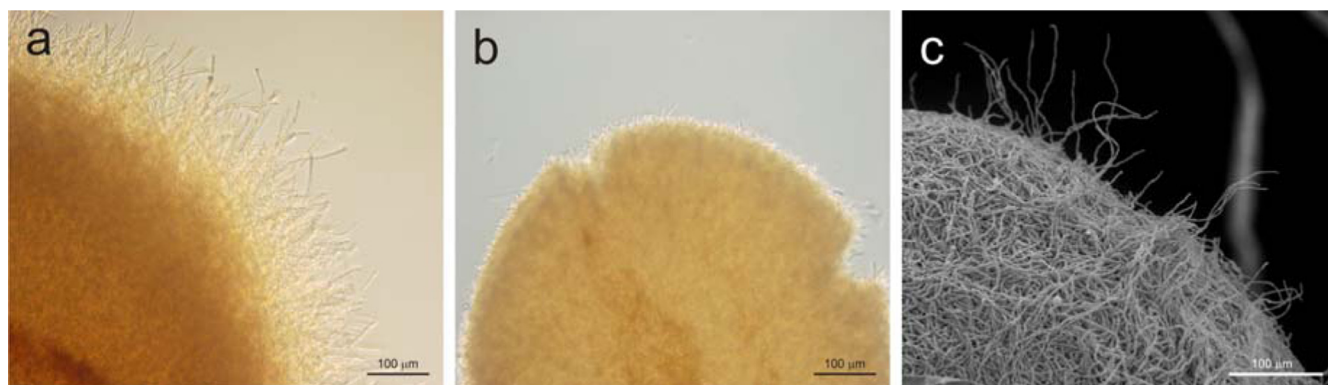


FIG. 2. Mycelial pellet morphologies of *W. sebi*. (a and b) Morphology of *W. sebi* mycelial pellet at 5% (a) and 20% (b) NaCl. (c) SEM micrograph of *W. sebi* mycelial pellet at 5% NaCl.

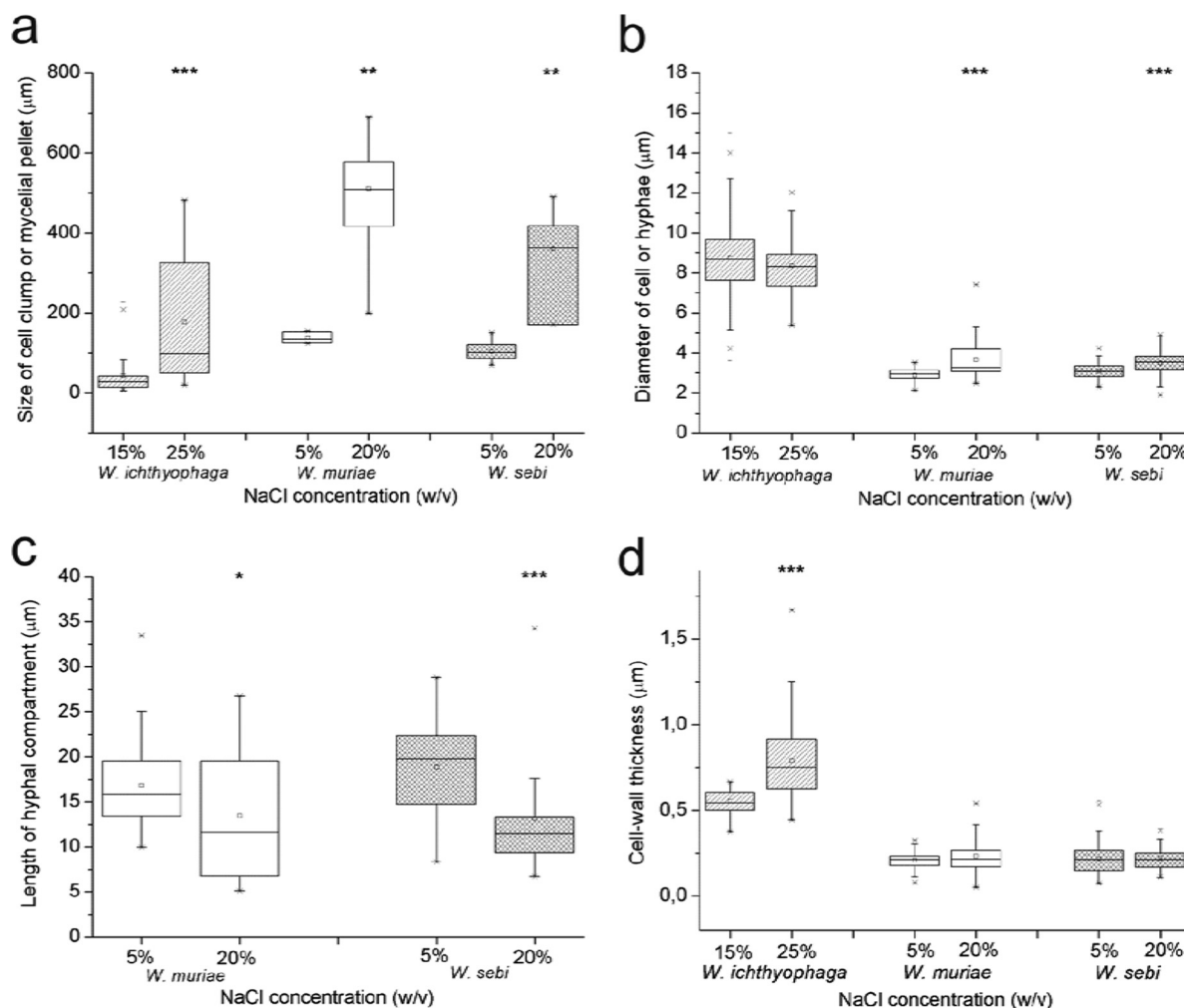


FIG. 3. Morphological characteristics of *Wallemia* spp. at the low and high salinities. Box plots of morphological characteristics show median (line in box), 75th percentile (upper edge of box), 25th percentile (lower edge of box), and minimum and maximum data values (whiskers) and outliers (\times). (a) Significant increases in size of the cell clumps (*W. ichthyophaga*, $n = 162$ [15%] and $n = 43$ [25%]) and mycelial pellets (*W. muriae*, $n = 5$ [5%] and $n = 12$ [20%]; and *W. sebi*, $n = 9$ [5%] and $n = 4$ [20%]) at the high salinities. (b) Nonsignificant changes in cell size of *W. ichthyophaga* ($n = 113$ [15%] and $n = 36$ [25%]) and significant increases in hyphal diameter of *W. muriae* ($n = 35$ [5%] and $n = 51$ [20%]) and *W. sebi* ($n = 61$ [5%] and $n = 42$ [20%]) at the high salinities. (c) Significant decrease for *W. muriae* ($n = 38$ [5%] and $n = 19$ [20%]) and *W. sebi* ($n = 29$ [5%] and $n = 21$ [20%]) in hyphal compartment length at the high salinity. (d) Significant differences in cell wall thickness of *W. ichthyophaga* ($n = 40$ [15%] and $n = 71$ [25%]) compared to *W. muriae* ($n = 87$ [5%] and $n = 84$ [20%]) and *W. sebi* ($n = 120$ [5%] and $n = 118$ [20%]) at the high salinities. Statistically significant differences are marked (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Morphology and cell wall ultrastructural changes in *W. ichthyophaga*. The *W. ichthyophaga* colonies on solid media at the low and high salinities also differed in size and color (Fig. 4a and b; Table 2). When grown in liquid medium, the most distinct morphological feature that distinguished *W. ichthyophaga* from *W. muriae* and *W. sebi* was the growth of *W. ichthyophaga* in multicellular clumps, composed of multiple spherical cells (Fig. 4c and d). These clumps were significantly larger at 25% NaCl (Fig. 3a). The high NaCl concentration resulted in a minor, but not significant, decrease in cell size (Fig. 3b). Scanning electron microscopy also revealed differences in the surface appearances of the multicellular clumps at the high salinity (25% NaCl). Here, the surfaces of the cells were more corrugated and less fully covered with EPS (Fig. 4e and f). Focused-ion-beam sectioning of these *W. ichthyophaga* clumps exposed the interior of the densely packed cells (Fig.

5). The image shown in Fig. 5 illustrates the presence of some EPS, which seals the spaces between the cells.

TEM of the *W. ichthyophaga* cells sampled in the exponential growth phase at 15 and 25% NaCl showed significant differences in the cell wall thicknesses (Fig. 3d). At the low salinity, the cell walls were uneven (Fig. 4g and h), with thicknesses up to $0.6 \mu\text{m}$, while at the high salinity the thicknesses increased to up to $1.6 \mu\text{m}$ (a 1.67-fold increase). A comparison of the median cell wall thicknesses of all three of the *Wallemia* spp. according to the Kruskal-Wallis test showed that at the high salinities, the cell wall thickness of *W. ichthyophaga* increased significantly ($P < 0.001$) compared to *W. muriae* and *W. sebi* (data shown in Fig. 3d). At both salinities, the thick cell walls of *W. ichthyophaga* were clearly structured in at least two distinct layers: a thinner, electron-dense outer layer, and a thicker, electron-translucent inner layer (Fig. 6a). At the low

W. ichthyophaga

15% NaCl

25% NaCl

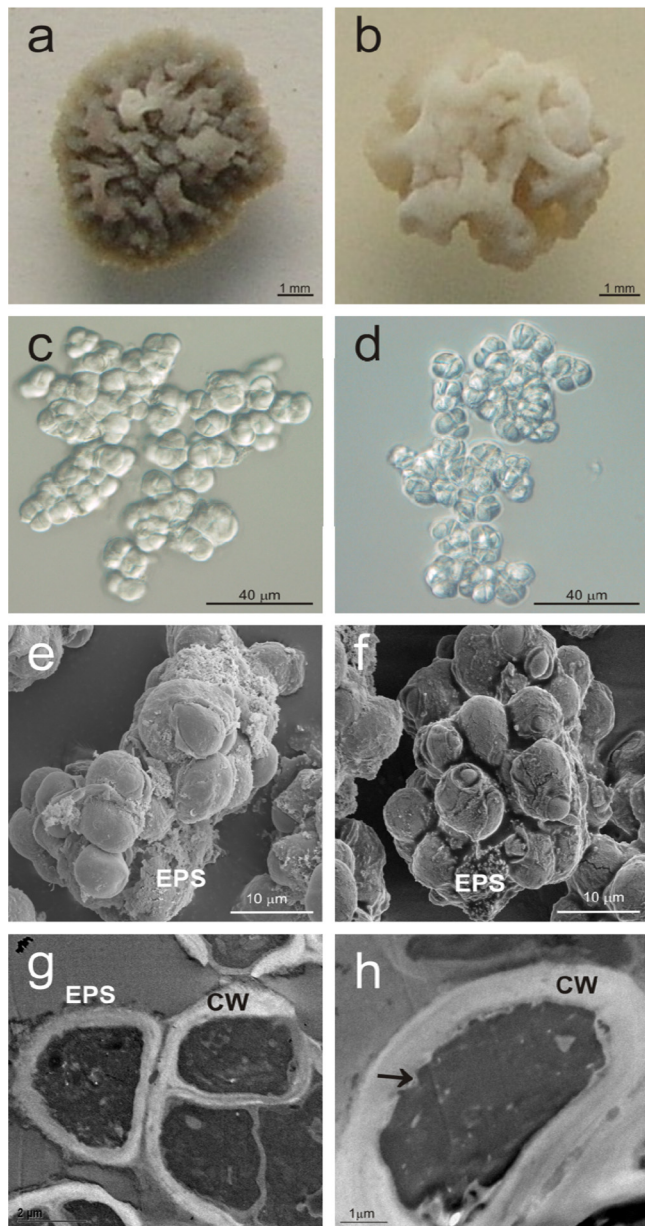


FIG. 4. NaCl effects on morphology of *W. ichthyophaga*. (a and b) Colony morphology on solid YNB at 15% (a) and 25% (b) NaCl. (c and d) Multicellular clumps, when cultured in liquid YNB media at 15% (c) and 25% (d) NaCl. (e and f) SEM micrographs of multicellular clumps, when cultured in liquid YNB with 15% (e) and 25% (f) NaCl. (g and h) TEM micrographs of cells, when cultured in liquid YNB with 15% (g) and 25% (h) NaCl. CW, cell wall; arrow, cell wall indentation.

NaCl concentration with *W. ichthyophaga*, the outer cell wall layer was covered with a clearly visible fibrous layer that was wide and well defined and composed of EPS (Fig. 4g). At both the low and the high salinities, there were also cell wall inden-

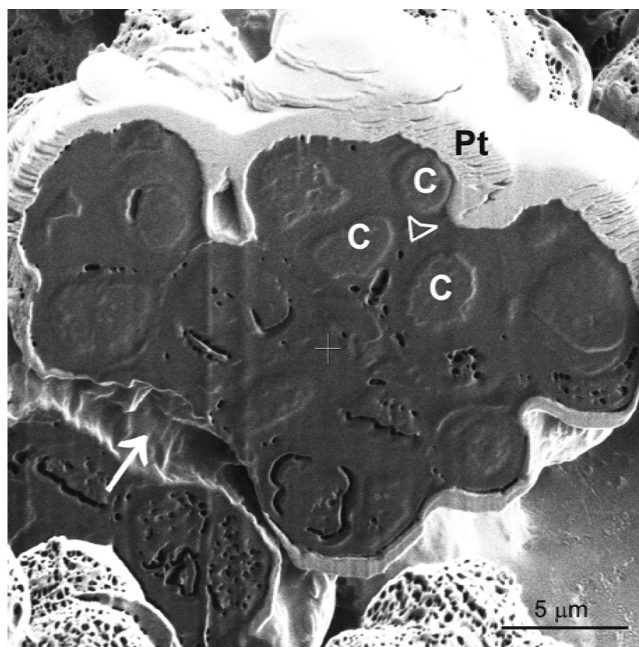


FIG. 5. Morphology of multicellular clumps of *W. ichthyophaga*. Secondary electron micrograph of focused-ion-beam sectioned multicellular clump of *W. ichthyophaga*, when cultured in liquid YNB with 25% NaCl. Pt, protective platinum layer against surface damage during focused-ion-beam sectioning; C, cells; arrow, space between two multicellular clumps; arrowhead, space between the cells in the clump.

tations, which were more numerous at high salinity (Fig. 4 h, marked with an arrow).

DISCUSSION

Halophilic and halotolerant fungi show a surprisingly rich diversity and abundance in natural saline environments, such as salt crystals, food preserved with high concentrations of salt, and solar salterns (8–10, 20), where biologically available water is limited. The ability to grow at a low a_w is apparent in 10 phylogenetically unrelated orders of fungi and is in most cases limited to a few species or to a single genus within an order. Moreover, the most representative of the xerophilic fungi belong to the division Ascomycota (16). However, the entire genus *Wallemia*, and therefore the entire phylogenetically old and separate order *Wallemiales* within the division Basidiomycota, is either xerophilic or xerotolerant. Two of the three *Wallemia* spp. show distinctly improved growth with NaCl as the solute (49). Their growth across increasing NaCl concentration ranges shown here confirm these previous findings of Zalar et al. (49). Indeed, *W. ichthyophaga* and *W. muriae* grew only in the presence of NaCl, whereas *W. sebi* grew on media without added NaCl. To our knowledge, *W. ichthyophaga* and *W. muriae* are the only fungal species that show a requirement for NaCl as a solute in the growth medium. Moreover, *W. ichthyophaga* was the only one of the three *Wallemia* spp. that also grew in saturated NaCl (i.e., 30% [wt/vol] NaCl).

The influence of high salinity on growth is seen in the growth characteristics of the *Wallemia* spp. (see Table 1). All three of these species had both lower specific growth rates and final

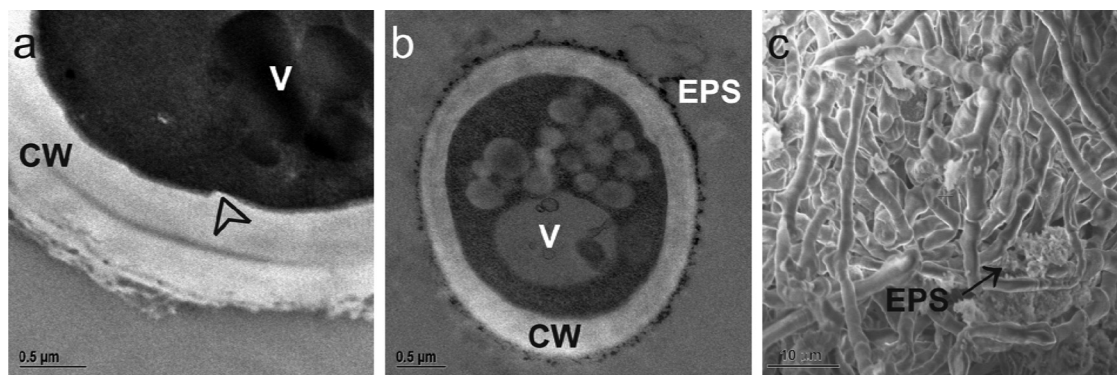


FIG. 6. Morphology details of *W. ichthyophaga*, *W. muriae*, and *W. sebi*. (a) TEM micrograph of structured cell wall of *W. ichthyophaga*, when cultured in liquid YNB with 25% NaCl. (b) Unpronounced EPS of *W. muriae* at 5% NaCl. (c) SEM micrograph of *W. sebi* at 20% NaCl. Arrowhead, cell wall indentation; arrow, EPS (c); CW, cell wall; V, vacuoles.

biomass yields at the high salinities. Growth at high salinities requires the adaptation of cellular metabolism. The reductions in the final biomass yields indicate the high energy demands of life at high NaCl concentrations, even in such well-adapted species (36). However, the growth rates and the final biomass yields of *W. ichthyophaga* were higher at both the low and the high salinities than those of *W. muriae* and *W. sebi*. This suggests better adaptation of *W. ichthyophaga* to high NaCl concentrations in the growth medium.

Our data demonstrate that NaCl concentrations have an impact on the cell morphology of the *Wallemia* spp. At a high salinity, the hyphal compartments in both *W. muriae* and *W. sebi* were thicker and shorter compared to what we observed at the low salinity. Such changes of the cell morphology resulted in changed size and shape of the mycelial pellets. The pellets of both *W. muriae* and *W. sebi* were small and regularly shaped at the low salinity, whereas at the high salinity, they were larger and variable in shape (Fig. 2). Shortening and thickening of the hyphal compartments and similar changes in pellet morphology have also been seen in less salt-adapted fungi, such as in the halotolerant *Aspergillus repens* when grown on medium with 12% NaCl (wt/vol) (25). At high salinity, we noted that the mycelial pellets of both *W. muriae* and *W. sebi* were less pigmented. In previous studies, a series of unique pyrrolylpolypene pigments of *W. sebi* were isolated and characterized (2–4), but no studies exist on the effects of high salinities on the pigment expression in *Wallemia* spp. Although pigmentation can protect cells from various harmful conditions and agents (11, 33), we would assume that the synthesis of these pigments is reduced at the high salinity due to high energy requirements (36).

In contrast to the mycelial growth of *W. muriae* and *W. sebi*, *W. ichthyophaga* grows in multicellular clumps, or sarcinalike structures (49), composed of multiple spherical cells. These clumps were significantly larger at the high salinity, whereas the sizes of cells in the clumps did not change significantly. The ability of microorganisms to organize into multicellular communities, as shown for *W. ichthyophaga*, can greatly enhance their survival in natural stressful environments as was proposed for yeast populations (37). Similar multicellular structures have also been seen at high salinities in the phylogenetically distant, extremely halotolerant ascomycetous black yeasts, such as *Hor-*

taea werneckii (T. Kogej and N. Gunde-Cimerman, unpublished data), *Phaeothea triangularis* (15, 48), and *Trimmatostroma salinum* (27), all of the order *Dothideales*. The ability to grow meristematically, the common feature of black yeasts at the high salinities, is hypothesized to enhance the ability to survive under conditions of stress (46), such as a high concentration of NaCl (47). Thus, we can conclude that multicellular clumps of *W. ichthyophaga* could also be a mode of protection at the high salinity.

The surface structures of the multicellular clumps of *W. ichthyophaga* and the morphological characteristics of their interiors were studied by FIB/SEM. FIB/SEM represents an upgraded imaging approach for biological samples when surface morphology and subsurface structures are being investigated at the same time across a range of magnifications (17, 22). The use of FIB/SEM techniques has revealed that the cells of *W. ichthyophaga* are densely packed into multicellular clumps, since a dense homogenous structure appeared after sectioning. If the cells were just loosely attached to each other, there should be gaps seen between the cells after FIB/SEM imaging (35). The presence of EPS was also seen by SEM in all three of the *Wallemia* spp. at the low and high salinities. The ability to produce a certain amount of EPS is also a morphological feature of rock-inhabiting black fungi, in which EPS is involved in the protection against cycles of desiccation, freezing, and thawing (42). It thus appears that EPS might also have a protective function.

TEM shows that in all three *Wallemia* spp., the cell wall is multilayered, or at least bilayered, at all salinities. It consists of a thick electron-translucent inner layer and a thin electron-dense outer layer. In ascomycetous fungi, the inner layer provides mechanical strength and attachment sites for the proteins that form the outer layer. The data on less-investigated basidiomycetes also suggest that the outer, electron-dense, cell wall layer is proteinaceous (14).

In the present study, increases in cell wall thickness are shown to correlate with successful growth at a low a_w , especially in *W. ichthyophaga* (Table 1; Fig. 3d). It is noteworthy that at the low salinities, the cell walls of cells in the multicellular clumps of *W. ichthyophaga* were threefold thicker than the cell walls of the hyphae of both *W. muriae* and *W. sebi*. At the high salinities, the hyphal cell walls of both *W. muriae* and

W. sebi barely increased in thickness, whereas the thickness of the *W. ichthyophaga* cell walls increased significantly ($0.8 \pm 0.2 \mu\text{m}$ at the high salinity as seen in Fig. 3d). Similarly thick cell walls (0.5 to $1.0 \mu\text{m}$) were reported for halophilic black yeasts of the genus *Trimmatostroma* exposed to high salinities (27) and for the marine fungus *Dendryphiella salina* (12). Clipson et al. (12) proposed that the thickened cell wall acts as a fungal survival mechanism in extremely saline environments. However, a thickened cell wall is not a general fungal response to salt stress, since certain moderately halotolerant fungi, such as *Aspergillus flavus* and *Penicillium roquefortii*, show decreased cell wall thickness under 8% NaCl salt stress, with an increased thickness of the cell membranes seen instead (1). We have also seen a decrease in cell wall thickness with 17% NaCl in the moderately halotolerant black yeast *Aureobasidium pullulans*, from $0.20 \mu\text{m}$ in nonsaline growth medium to $0.15 \mu\text{m}$ (Kogej and Gunde-Cimerman, unpublished). This has led us to speculate that the thickness of the cell wall and the ability to increase the cell wall thickness at higher salinities is an important feature of halophilic fungi but not of halotolerant fungi.

A thickened cell wall is probably related to changes in the molecular composition. It can be a consequence of the branching degree of the polysaccharides (31), an elevated β -1,3-glucan level (31), incorporation of proteins into the cell wall as a result of changed glucan synthesis (43), or elevated chitin levels (24, 26, 31, 38). The mechanisms involved in the cell wall thickening of the *Wallemia* spp. remain to be elucidated in further studies. However, our preliminary data on the differential expression of *W. ichthyophaga* genes at low and high salinities might hint at the cross connections between cell wall restructuring processes and fungal osmoadaptation. Our data have shown a pronounced increase in the expression of the cellulase/exo-1,3- β -glucanase gene (i.e., EXG1) that correlate with increasing salinity (C. Gostinčar and N. Gunde-Cimerman, unpublished data). Exo-1,3- β -glucanase is involved in the hydrolysis of glucosidic linkages of cell wall β -1,3-glucans and thus participates in morphogenetic processes. It has also been shown in *Saccharomyces cerevisiae* that the expression of the EXG1 gene is upregulated when Pbs2, the HOG pathway mitogen-activated protein kinase (MAPK), is overexpressed (23). The partial amino acid sequences of Hog1-like MAPKs from *Wallemia* spp. have already been determined and compared to the Hog1 protein from *S. cerevisiae* (30). It remains to be determined, however, whether in *W. ichthyophaga* there is a pathway analogous to the HOG pathway in *S. cerevisiae* that regulates the osmoadaptation processes.

In conclusion, the combination of light, FIB/SEM, and TEM has demonstrated various morphological changes at high salinity in three *Wallemia* spp. At the high salinities, the common morphological feature was the increase in the sizes of both the multicellular clumps of *W. ichthyophaga* and the mycelial pellets of *W. muriae* and *W. sebi*. However, there were marked differences on the cellular level. In both *W. muriae* and *W. sebi*, the elongated hyphal cells were shorter and thicker, which resulted in a changed surface-to-volume ratio. The decreased surface-to-volume ratio was mainly a consequence of decreased surface area, since the volume of the hyphal cells remained unchanged. In contrast, the spherical cells of *W. ichthyophaga* retained their size and thus the surface-to-volume ratio at the high salinity, but the thickening of the cell walls

resulted in decreased functional volume of the cells. The thick cell wall might be important as an armor against changes of osmotic pressure, since it provides a mechanical protection against hyposaline stress at dilution conditions. In the natural environment of these fungi, such as solar salterns, changing osmotic conditions as a consequence of change of salinity are a constant threat. In general, the mycelial form, as in *W. muriae* and *W. sebi*, enables penetration of solid substrata and colonization of a fixed spatial domain (13). We might speculate that in addition to the growth in multicellular clumps, the spherical form of the cells of *W. ichthyophaga* has a role in the adaptation to growth in hypersaline water of solar salterns. The sphere has a minimal surface-to-volume ratio, and this cell shape could be one of the important adaptations for growth of *W. ichthyophaga* in saturated NaCl (i.e., 30% [wt/vol] NaCl). At high salinities, the dominant fungi in the hypersaline waters of the salterns, the halophilic black yeasts *H. werneckii*, *P. triangularis* (15, 48), and *T. salinum* (27), phylogenetically distant from *Wallemia* spp., all grow meristematically and have spherical cells, a finding which supports this conclusion.

The overall morphological changes seen in *Wallemia* spp. correspond to the phylogenetics based on the ITS ribosomal DNA gene (49). *W. ichthyophaga* clearly shows its halophilic character, which might represent an evolutionary "cul de sac" of this ecologically very specialized and unique species. Due to its character, we believe that *W. ichthyophaga* is a particularly suitable model organism for the study of halophily in eukaryotes. Intensified studies of halophilic eukaryotic microorganisms can give us clues toward our understanding of stress responses and of the targets, processes, and networks involved in the complex field of salt tolerance.

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