

Extremely Halotolerant and Halophilic Fungi Inhabit Brine in Solar Salterns Around the Globe

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Summary

For a long time halotolerant and halophilic fungi have been known exclusively as contaminants of food preserved with high concentrations of either salt or sugar. They were first reported in 2000 to be active inhabitants of hypersaline environments, when they were found in man-made solar salterns in Slovenia. Since then, they have been described in different salterns and salt lakes on three continents. The mycobiota that inhabit these natural hypersaline environments are composed of phylogenetically unrelated halotolerant, extremely halotolerant, and halophilic fungi, which are represented not only by species previously known only as food contaminants, but also by new and rare species. The dominant representatives are different species of black yeast-like and related melanized fungi of the genus *Cladosporium*, different species within the anamorphic *Aspergillus* and *Penicillium*, and the teleomorphic *Emericella* and *Eurotium*, certain species of non-melanized yeasts, and *Wallemia* spp. Until the discovery and description of indigenous saltern mycobiota, the physiological and molecular mechanisms related to salt tolerance in eukaryotic microorganisms were studied using salt-sensitive model organisms. The most studied eukaryotic microorganism was *Saccharomyces cerevisiae*, which cannot adapt to hypersaline conditions. Species like *Debaryomyces hansenii*, *Aureobasidium pullulans*, *Hortaea werneckii* and *Wallemia ichthyophaga*, which have now been isolated globally from natural hypersaline environments, represent more suitable model organisms for the study of halotolerance in eukaryotes. Such studies in these species, and particularly with the extremely halotolerant *H. werneckii* and obligately halophilic *W. ichthyophaga* have continued to unravel the different strategies that these microorganisms can use to cope with the problems of ion toxicity and low water activity. The focus of this review is to present the main species of fungi inhabiting solar salterns around the world and the most suitable model fungi to study adaptations to life at high salinity.

Key words: fungi, black yeasts, *Aspergillus*, *Cladosporium*, *Emericella*, *Hortaea*, *Penicillium*, *Phaeothea*, *Trimmatostroma*, *Wallemia*, salterns

Introduction

Extreme environments, as exemplified by hypersaline ones, have long been considered to be populated almost exclusively by prokaryotic organisms. The first studies on the presence of bacteria in hypersaline envi-

ronments appeared as early as 1914 (1,2). More recent studies have been dedicated to halophilic bacteria and archaea (3–5) and the alga *Dunaliella salina* (6). For more than 80 years after the discovery of halophilic bacteria, fungi that can survive in environments with low water activity (a_w) were only known as contaminants of food

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that had been preserved using high concentrations of salt or sugar (7). It was a general belief in mycology that these foodborne fungi have a general xerophilic phenotype (8), which is determined by the water potential of the medium, rather than by the chemical nature of the solute (9,10). Therefore, they were described as xerophilic or xerotolerant if they grew well on substrates with $a_w \leq 0.85$, which corresponds to 17 % NaCl or 50 % glucose in their growth medium. The term halophilic for fungi was introduced as late as 1975 for a few foodborne species that showed superior growth on media with NaCl as the controlling solute (11).

Due to the limited knowledge of halophilic fungi and their adaptive abilities, no fungal research was conducted in natural hypersaline environments. Prior to 2000, when fungi were first described as active inhabitants of solar salterns (12), only a few reports describing the isolation of fungi from natural moderately saline environments, such as salt marshes (13), saline soil (14), and sea water (15) were available. Their contribution to hypersaline microbial communities was first documented for the 700-year-old seasonal salterns in Sečovlje, Slovenia (16), and then later confirmed in many eutrophic salterns around the world, as well as in hypersaline lakes such as the Dead Sea in Israel and the Great Salt Lake in the USA (17). Based on these diversity and population studies, fungi are now recognized as an integral part of the indigenous microbial communities in natural hypersaline habitats.

Isolation, Detection and Identification of Fungi in Salterns

Fungi were initially isolated from hypersaline water samples taken from seasonal solar salterns Sečovlje along the northern Adriatic coast, on the border between Slovenia and Croatia (12). The hypersaline waters were sampled throughout the season, from May to October, and the fungi were isolated by filtration of the water, microbial baiting and spreading of biofilms covering the surface of the brine. Fungal population dynamics was then followed using filtration. Selective media with lowered a_w as a result of high fractions of NaCl (from 17–32 %) or sugars (from 50–70 %) were incubated with added antibiotics (to prevent bacterial growth) from several days up to several weeks. All of the isolated fungi were examined for their ability to grow at 17 % NaCl, as the criterion for halophily (12). These fungi appeared in distinct seasonal peaks, and reached 40 000 CFU/L on enumeration media. These peaks primarily correlated with the highest levels of phosphorus and nitrogen, followed by dissolved oxygen concentration, a_w , pH and year of sampling (12,18).

These initial studies were later supplemented with the isolation of fungi from the crystallizers of different salterns that operate throughout the year on three continents, including the salterns along the Red Sea coast in Israel (Eilat), along the Mediterranean coast in Spain (Santa Pola, and the Ebro River Delta) and France (Carmargue), along the Atlantic coast in Namibia (Skeleton Coast), and along the coasts of the Dominican Republic (Monte Cristy), Puerto Rico (Fraternidad) and Portugal

(Samouco). Fungi were also isolated from the Dead Sea (Ein Bokek, Ein Gedi), the Great Salt Lake (Utah, USA) and the Enriquillo Salt Lake (Dominican Republic) (19). The presence of fungi was not only documented in brine, but also on wood immersed in brine (20), on the surface of halophytic plants (Zalar *et al.*, unpublished data), and most recently, in tropical microbial mats (21–23). Many of the halotolerant species commonly found in salterns were also found in Arctic glacial ice and other water-based Arctic environments, due to low a_w as the common critical parameter in both environments (24).

Since 1998, 22 species of fungi have been either described as new in science or newly accommodated from hypersaline environments. They include three species of the genus *Wallemia*, *i.e.* *W. sebi*, *W. muriae* and *W. ichthyophaga*, which were accommodated into the newly described class Wallemiomycetes and order Wallemiales (25), two new species of the genus *Emericella*, *i.e.* *E. appendiculata* and *E. stella-maris* (26), black yeast *Trimmatostroma salinum* (27) and twelve new species of the genus *Cladosporium*, *i.e.* *C. dominicanum*, *C. fusiforme*, *C. halotolerans*, *C. herbarioides*, *C. psychrotolerans*, *C. ramotenellum*, *C. salinae*, *C. spinulosum*, *C. subinflatum*, *C. subtilissimum*, *C. tenellum* and *C. velox* (28,29). The species *Aureobasidium pullulans* was redefined as four varieties, one of them, *i.e.* *A. pullulans* var. *pullulans*, was frequently detected in hypersaline water environments (30). Additionally, two new species of *Candida*, *i.e.* *C. candida* galli-like and *C. pseudorugosa*-like (31), and *Eurotium halotolerans* were isolated from hypersaline waters of different salterns worldwide (32).

The presence of fungi in the salterns was confirmed also by their chemical lipid signature in the environment. Examination of the lipid composition of the dominant melanized yeast-like fungi revealed sterols as the most distinct lipid fraction. Ergosterol dominated all of the distributions, but the major differences between species related to the subordinate sterols. The characteristic 24-methylcholest-7-en- β -ol and 24-methylcholesta-7,24(28)-dien- β -ol were described in significant proportions in the water column particles and sediments of gypsum and halite precipitation ponds, providing a specific signature for the presence of these fungi (33).

Although the diversity of fungi inhabiting hypersaline waters is surprisingly high, certain genera and species of fungi dominate almost all of the investigated environments. They are divided in three main groups: (i) halotolerant and halophilic fungi that have been primarily isolated on selective media containing different concentrations of NaCl, (ii) xerotolerant fungi that grow on both sugar and saline media, and (iii) sporadic airborne isolates that 'contaminate' low a_w media and are present in low numbers.

Disregarding the medium from which they were selected, only fungi that can grow *in vitro* in 3 M NaCl have been further identified and analyzed. Some of the typical representatives of this group show adaptive xerotolerance/halotolerance, and these are represented by different species of the genera *Aspergillus*, *Penicillium* and *Eurotium*, and by some species of the genus *Cladosporium*.

The dominant halotolerant and halophilic fungi were almost exclusively isolated on selective media with high

concentrations of NaCl. This group of fungi displayed halophilic behaviour that differed both from that of the majority of halophilic prokaryotes as well as from the xerotolerant/halotolerant fungi mentioned earlier. With few exceptions, the fungi in this group did not require NaCl for viability, as they were able to grow and adjust to the whole salinity range, from freshwater to almost saturated NaCl solutions (34). Their growth *in vitro* was optimal across a broad salinity range, from 5 to 17 % NaCl, and they have been regularly isolated from global environments at salinities of above 10 % NaCl (35). To accommodate this particular type of adaptation, a new term of 'extremely halotolerant fungi' was introduced (24,34). These were represented primarily by black yeast-like fungi and related species from the melanized filamentous genera *Cladosporium* and *Alternaria* (36). The term halophilic is thus now used only for the few species of fungi that have an obligate requirement for NaCl in the growth medium, such as two species of the genus *Wallemia*, i.e. *W. muriae* and *W. ichthyophaga* (25).

Halotolerant and Halophilic Fungi in Hypersaline Environments

Saltern mycobiota

At present there are a total of 140 orders of fungi known (37); however, growth at decreased a_w is in most cases limited to a few species or to a single genus of only 10 genera. Before studies of fungi in natural hypersaline environments had started, representatives of these groups were described almost exclusively as xerotolerant or xerophilic foodborne fungi, with an unknown primary ecological niche. Since then, besides many foodborne species, also new and rare species have been isolated (12). We now know that in multipond solar salterns, the pre-crystallization and crystallization ponds in particular harbour a surprisingly rich diversity and abundance of phylogenetically unrelated halophilic and halotolerant fungi, regardless of the geographic locality, and with a relatively consistent and stable species composition (12,17,18,25, 26,28,31,32). The mycobiota in hypersaline waters around the world include meristematic melanized yeast-like fungi, the so-called black yeasts, different related species of the genus *Cladosporium* (12,28,32), non-melanized yeasts (31), the filamentous genera *Wallemia*, *Scopulariopsis* and *Alternaria* (19,25), and different species of the anamorphic genera *Aspergillus* and *Penicillium*, including some of their teleomorphic genera *Eurotium* and *Emericella* (17,18). The main groups of fungi inhabiting the salterns around the world are presented in Fig. 1.

Aspergillus, *Penicillium* and related teleomorphic genera *Emericella* and *Eurotium*

The isolation and identification of 60 different species of *Aspergillus* and *Penicillium* confirm the diversity of these genera in oligotrophic and eutrophic hypersaline waters around the world. Strains were identified according to their morphological characteristics and extrolite profiles, and when needed, by sequencing of the relevant molecular markers. Since the species *Aspergillus niger*, *Eurotium amstelodami* and *Penicillium chrysogenum* were detected with the highest frequencies at all of the sampled

sites, they represent the pan-global stable mycobiota in hypersaline environments. *Aspergillus sydowii* and *Eurotium herbariorum* were also quite evenly distributed, while *Aspergillus candidus*, which was abundant, was distributed more locally. These species and others, less frequently present, are known producers of mycotoxins and other secondary metabolites, thus their byproducts can potentially accumulate in evaporated brine. Consequently, marine salt used for consumption can be a potential source of foodborne fungi and their byproducts (18).

Non-melanized yeasts

The dominating yeasts in hypersaline environments are melanized. However, non-melanized yeasts have also been isolated from salterns and hypersaline lakes worldwide, although in much smaller numbers. Among the isolates obtained from hypersaline waters were: *Pichia guilhermondii*, *Debaryomyces hansenii*, *Yarrowia lipolytica* and *Candida parapsilosis*, which are known contaminants of low water activity food. Additional isolated species were: *Rhodospiridium sphaerocarpum*, *R. babjevae*, *Rhodotorula laryngis*, *Trichosporon mucoides*, and a new species resembling *Candida glabrata*, which had previously not been known for their halotolerance and were identified for the first time in hypersaline habitats. Moreover, the ascomycetous yeast *Metschnikowia bicuspidata*, known to be a parasite of the brine shrimp, was isolated as a free-living form from the Great Salt Lake brine. Surprisingly, non-melanized yeasts, provisionally named *Candida atmosphaerica*-like and *Pichia philogaea*-like, were isolated from the extremely hostile waters rich in magnesium chloride (bitterns) (31).

The genus Cladosporium

The genus *Cladosporium* (anamorphs of *Davidiella*) represents an important group of extremophilic fungi within Capnodiales. This genus has a cosmopolitan distribution, is ubiquitous decomposer of dead plant material (38), and is therefore commonly found in indoor and outdoor air. However, some species that have been described as new have been consistently isolated from salterns and salty lakes worldwide, where they represent the most common and frequent fungal taxa (12). Initially, they were considered as airborne contaminants and identified as *Cladosporium sphaerospermum* (39). Subsequent taxonomic analyses revealed a complex of twelve new species that inhabit brine in the salterns, but many of them are important cosmopolitan species with wider ecological distributions (28,29). For example, *C. halotolerans* represents a saprobic species, isolated from hypersaline saltern water of temperate region, but also from subtropical climates. It is frequently encountered in indoor environments, Arctic ice and biomats, as a contaminant in lesions of humans and animals, plants, rock, conifer wood, and mycorrhizal roots. It is assigned as a circumglobal species, found in Africa (Namibia, South Africa), the Arctic, Asia (India, Israel, Turkey), Australasia (New Zealand), Europe (Belgium, Bosnia and Herzegovina, Denmark, Germany, Italy, Russia, Slovenia, Spain, Sweden, Switzerland, UK), North America (USA) and Central and South America (Brazil, Dominican Republic). In recent years, the genus has received a lot of attention and many new species have been described also from other environments (40).

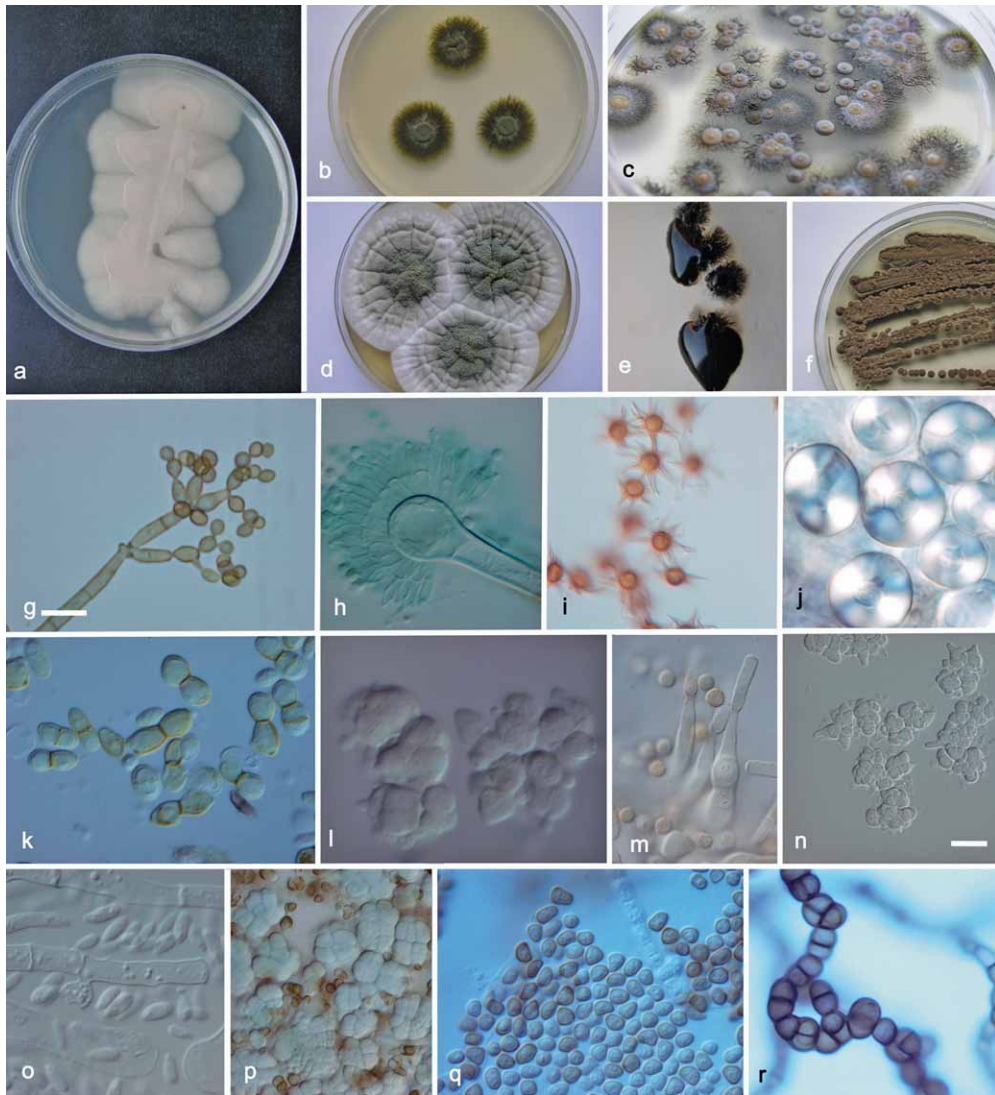


Fig. 1. Morphology of fungal colonies (a–f) and microscopic structures of halotolerant and halophilic fungi (g–r): a) *Aureobasidium pullulans* var. *pullulans*, b) *Cladosporium sphaerospermum*, c) *Hortaea werneckii*, d) *Emericella stella-maris*, e) *Phaeotheca triangularis*, f) *Wallemia ichthyophaga*, g) top of *Cladosporium herbarum* conidiophore; h–j) *Emericella stella-maris*: h) *Aspergillus* anamorph, i) ascospores, j) Hülle cells; k and l) *Hortaea werneckii*: k) budding cells, l) meristematic clumps; m and n) *Wallemia ichthyophaga*: m) conidiophores, n) meristematic clumps; o) *Aureobasidium pullulans* laterally budding hyphae; p and q) *Phaeotheca triangularis*: p) meristematic clumps, q) released endoconidia; r) *Trimmatostroma salinum* meristematic hyphae. Scalebars: g–m, o, q, and r: 10 µm; n and p: 20 µm

Black yeasts as the dominant fungi in salterns around the world

The dominant fungal inhabitants of salterns are ascomycetous black yeasts. Their main halophilic and halotolerant representatives belong to the orders Capnodiales and Dothideales, both of which are known for their xerotolerant tendencies. They populate hypersaline coastal salterns worldwide (32), and include a large number of species that can tolerate different physicochemical extremes and grow as epilithic or cryptoendolithic species at high or low temperatures (41,42), including in Arctic glacier ice (43). These fungi are practically unknown in non-natural environments, and of particular note, they have not even been isolated from salted food products.

Black yeasts are polymorphic fungi with the ability to develop yeast-like, filamentous or meristematic growth. They produce melanin pigment in their hyphae, yeast-

-like cells, conidia and clump-like colonies. In water or under stressed conditions, they characteristically consist of isodiametrically dividing cells, while hyphal growth is mainly seen on solid media (44).

Black yeasts are notoriously difficult to identify at the morphological level, due to their above-described morphological plasticity and the polymorphism of their different sinanamorphic stages (45). Therefore, their identification has to be complemented by molecular methods. The dominant halophilic species of black yeasts are represented by *Hortaea werneckii* (46), *Phaeotheca triangularis* (46,47), *Aureobasidium pullulans* (46) and a new species *Trimmatostroma salinum* (27).

Amongst the saltern mycobiota that have been described so far, three species have been selected as model organisms for in-depth studies of the molecular mechanisms that allow growth at high NaCl concentrations: the

halotolerant black yeast *Aureobasidium pullulans* (46), the extremely halotolerant black yeast *Hortaea werneckii*, and the halophilic *Wallemia ichthyophaga*. At present, *H. werneckii* is the most studied eukaryotic organism at the level of its molecular response to high NaCl concentrations (34).

The genus *Wallemia*

The order Wallemiales (and class Wallemiomycetes) is a 250-million-year-old sister group of Agaricomycotina according to phylogenomic analysis. It was recently introduced to accommodate the single genus *Wallemia*, a separate and old phylogenetic line within the Basidiomycota (25,48). Until recently, this genus contained only the species *Wallemia sebi*. However, based on taxonomic analyses of isolates from sweet, salty and dried food (49) and from salterns and salt lakes (25,50), three species have now been described: *W. sebi*, *W. muriae* and *W. ichthyophaga* (25). The last two require additional solutes in their media for growth, such as NaCl or sugar. *Wallemia ichthyophaga* is presently the most halophilic fungus known to date. It shows preference for media supplemented with salt over media with high concentrations of glucose and it grows only between 10 % by mass per volume of NaCl and saturation (32 % NaCl), equivalent to a_w of 0.77. Such obligate halophily is common in archaea, but unique in fungi. *Wallemia ichthyophaga* differs from the other two *Wallemia* species in its ability to form multicellular, sclerotium-like meristematic clumps, resembling those observed in the phylogenetically distant black yeasts.

Model Fungi for the Study of Adaptations to Life at High Salinity

Hortaea werneckii

Hortaea werneckii was originally known as the causative agent of human tinea nigra, a characteristic type of colonization of salty human hands (51). It is now known that it is widely dispersed in seawater-related environments (46) and that it is the dominant species in hypersaline evaporation ponds (12,46). Successive years of investigations of mycobiota in Slovenian solar salterns have revealed seasonal peaks of *H. werneckii*, which correlate primarily with high nitrogen and phosphorus concentrations.

As with nearly all of the extremophilic species within Capnodiales, *H. werneckii* is heavily melanized, has a thick cell wall, and at high salinity shows a meristematic, isodiametric type of thallus expansion, leading to cell clumps, which provides high resistance to increased salinity, changes in temperature and desiccation (52). This morphology is interpreted as optimization of its surface to volume ratio. This type of sclerotium-like protective structure is necessary for survival in extreme environments that have multiple stress factors. At lower salinities, the morphology of *H. werneckii* is characteristically polymorphic (41,44,46,53).

As with other adapted organisms, the extremely halotolerant black yeast *H. werneckii* reacts to extreme environmental conditions by physiological and metabolic changes that lead to adaptive responses. These responses depend partly on the nature of the stress-producing agent and partly on the mechanisms available to the

system. *H. werneckii* is naturally adapted to fluctuating salt concentrations, to the extent that it can grow without salt and up to NaCl saturation. It thus represents a suitable and highly relevant organism for the study of adaptive cellular responses to environmental perturbations, such as life in its primary natural habitat: the brine in solar salterns around the world.

Indeed, *H. werneckii* is to date the most studied extremely halotolerant eukaryotic model organisms (54,55). Over the last ten years, it has been demonstrated that *H. werneckii* has distinct mechanisms of adaptation to hypersaline environments that have not been seen with either salt-sensitive or moderately salt-tolerant fungi (34). The most relevant physiological characteristics are the properties and composition of plasma membrane (56,57), osmolyte composition and accumulation of ions (58–60), and structure and melanization of the cell wall (61,62).

Wallemia ichthyophaga

Wallemia ichthyophaga is a rare and still insufficiently studied basidiomycetous fungus from the ancient genus *Wallemia* (Wallemiales, Wallemiomycetes) that was first isolated from the evaporation ponds of Sečovlje solar saltern (Adriatic coast, Slovenia) (25). It deserves special attention since it has an obligatory requirement for at least 10 % of NaCl (by mass per volume) in the growth medium and grows also in saturated NaCl solution. This obligative salt requirement is extraordinary not only in the phylum Basidiomycota, but in the whole fungal kingdom. The optimum salinity range for growth of *W. ichthyophaga* in both solid and liquid culture is between 15 and 20 % (by mass per volume) of NaCl. Given that the genome of the only obligative fungal halophile, *W. ichthyophaga*, has been sequenced recently and that we standardized its cultivation and uncovered the basics of its osmoadaptation, *W. ichthyophaga* should well represent a novel model organism for studies of halophily in eukaryotes (48,63–65).

Adaptive Mechanisms

The compatible solute strategy

Exposure of fungi to high salinity represents two different environmental stimuli: one is osmotic stress, and the other is ionic stress. In non-adapted organisms, hyperosmotic stress triggers water efflux from the cell that results in the reduction of the turgor pressure and dehydration of the cytoplasm, thereby increasing the solute concentration in the cytosol. Ionic stress on the other hand causes ions (e.g. Na⁺) to enter the cytoplasm, leading to a further increase in the ion concentration and the subsequent damage of the membrane systems, as well as of cellular proteins. Fungi adapted to life at low a_w by accumulating the compatible solutes that do not interfere with the vital functions of their cellular proteins as their main survival strategy, to counteract changes in turgor pressure. They also need to maintain their intracellular concentrations of Na⁺ below toxic levels (62). Most halophilic bacteria accumulate these compatible solutes, such as ectoine, glycine betaine, glutamate and proline, while the accumulation of glycerol is well known in the alga *Dunaliella salina* and in most

fungi (66,67). In *D. salina*, the accumulated glycerol can represent up to 70 % of their cell mass when they are growing in salt-saturated brine. Glycerol is also the main compatible solute in halotolerant *A. pullulans*, extremely halotolerant *H. werneckii*, and halophilic *W. ichthyophaga*, as well as in other halotolerant fungi. Due to the specific meristematic growth in multicellular clumps of these fungi, estimation of their glycerol content is harder to obtain (58,62,68). It has been shown that glycerol in combination with trehalose and other polyols can maintain positive turgor pressure at high salinity in moderately salt-tolerant yeast species, like *Debaryomyces hansenii*, *Candida versatilis*, *Rhodotorula mucilaginosa* or *Pichia guilliermondii* (69–71). In the black yeast *H. werneckii*, this can be complemented at lower salinities by mycosporine-glutaminol-glucoside, a mycosporine that was primarily known as being involved in fungal sporulation and UV protection (72), and at higher salinities by other polyols, such as erythritol, arabitol and mannitol (60,61). In *W. ichthyophaga* glycerol is complemented with minor amounts of arabitol and only trace amounts of mannitol (65).

Ion homeostasis

Physiological studies on *H. werneckii* and *W. ichthyophaga* showed that in contrast to the moderately salt-tolerant marine yeast *D. hansenii*, which accumulates more Na⁺ than *Saccharomyces cerevisiae* (73), they maintain very low intracellular K⁺ and Na⁺ levels even when grown in the presence of 4.5 M NaCl. This suggested that *H. werneckii* can effectively extrude and prevent the influx of Na⁺ ions. Indeed, identification of the two salt-responsive P-type (ENA-like) ATPases (74) and their salt-dependent activities is in line with this concept. Thus, in response to elevated NaCl concentration, *H. werneckii* can be classified as a Na⁺ extruder with an intricate compatible solute strategy, which has been revealed by genomic data (55). *Wallemia ichthyophaga*, on the other hand, lacks most cation transporters, present in the genome of *H. werneckii*, therefore its main strategy appears to be prevention of ion entry, as a result of extremely thickened cell walls (63–65).

Cell wall structure and pigmentation

The cell wall of black yeasts is melanized. It has been shown that in *H. werneckii* this melanization depends on the salt concentration in the medium. When grown without NaCl, melanin granules are deposited in the outer layer of the cell wall, where they form a thin layer of melanin with separate larger granules. At optimal salinity, on the other hand, *H. werneckii* forms a dense shield-like layer of melanin granules on the outer side of the cell wall (60,61), which can mechanically stabilize the cell wall, thus counterbalancing the increased turgor pressure (60,61).

Wallemia ichthyophaga grows meristematically and forms characteristic compact multicellular clumps. When growing at higher salinity, a threefold thickening of the cell wall and an almost fourfold increase in the size of multicellular clumps occurs. The thick cell wall and compact cell clumps are thought to be important for the successful growth in extremely saline conditions (63,64).

Plasma membrane fluidity

As described above, the intracellular hyperaccumulation of glycerol as the main compatible solute at elevated concentrations of NaCl is an important physiological response of *H. werneckii*. However, glycerol has a high permeability coefficient for passage through lipid bilayers. As such, eukaryotic cells that use glycerol as a compatible solute need to combat the resulting loss of glycerol either by its accumulation using transport systems, which is energetically costly, or by changing the properties of their cell membrane, by increasing the sterol content or by reducing the membrane fluidity (75).

We have demonstrated that in comparison with the membranes of salt-sensitive and halotolerant fungi, the plasma membrane of *H. werneckii* is significantly more fluid over a wide range of salinities (56,57). In *H. werneckii*, the total sterol content of their plasma membrane remains largely unchanged with increased salinity (56), and thus their increased plasma membrane fluidity is the result of changes in the structure of the component phospholipids. It thus appears that by modifying the cell wall structure instead of directly lowering the membrane fluidity, *H. werneckii* can maintain high membrane fluidity even at high salinities. Thus, we hypothesize that at optimal growth salinities, the melanized cell wall of *H. werneckii* also helps it to retain higher concentrations of glycerol in the cells, despite the highly fluid membrane (76).

Genomes of halophilic fungi

In 2012 the first genome of a halophilic eukaryotic microorganism, the extremely halotolerant black yeast *Horataea werneckii*, originating from Slovenian salterns Sečovlje was sequenced (55). This genome was soon followed by the genome of the obligately halophilic fungus *Wallemia ichthyophaga*, also isolated in Sečovlje salterns (48). In both cases the genomic investigations were supplemented with analyses of transcriptomes.

The genome sequencing of *H. werneckii* explained much of its exceptional adaptability to osmotically stressful conditions. The most striking characteristic that may be associated with its extremely halotolerant lifestyle is the large genetic redundancy and the expansion of genes encoding metal cation transporters. Although no sexual state of *H. werneckii* has yet been described, a mating locus with characteristics of heterothallic fungi was found. The total assembly size of the genome is 51.6 Mb, larger than most phylogenetically related fungi, coding for 23 333 genes, which is almost twice the usual number of predicted genes. The genome appears to have experienced relatively recent whole genome duplication, and contains two highly identical gene copies of almost every protein. This is consistent with some previous studies that reported increases in genomic DNA content triggered by exposure to salt stress. In hypersaline conditions transmembrane ion transport is of utmost importance. The analysis of predicted metal cation transporters showed that most types of transporters experienced several gene duplications at various points during their evolution. Consequently, they are present in much higher numbers than expected. The involvement of plasma P-type H⁺ ATPases in adaptation to different concentrations of salt was indi-

cated by their salt-dependent transcription. This was not the case with vacuolar H⁺ ATPases, which were transcribed constitutively (55).

Sequencing of the genome of the *W. ichthyophaga*-type strain revealed its small size. The genome is among the smallest of known fungal genomes. It contains only 9.6 Mb, 1.67 % repetitive sequences and 4884 predicted protein-coding genes (a number in the range observed for *Escherichia coli*), which cover almost three quarters of the sequence. The genome does not contain a recognizable mating type locus and the species appears to be asexual. A few protein families are significantly expanded (the P-type ATPase cation transporters and hydrophobins, *i.e.* cell-wall proteins with multiple cellular functions), whereas most of them are contracted. Surprisingly, most genes and mechanisms that are involved in salt tolerance in other fungi do not appear to be substantially different in *W. ichthyophaga*. One of the unusual discoveries resulting from the genome and transcriptome analysis was the vast enrichment of hydrophobins, which contain an unusually large number of acidic amino acids and are also transcriptionally responsive to salt. Hydrophobins are of particular interest in biotechnology, pharmaceuticals and medicine due to their diverse applications (48).

Biotechnological potential

Studies of fungal models have contributed to the advances in the understanding of the molecular mechanisms that underlie the adaptive responses of extremely halotolerant and halophilic fungi, which appear as increasingly useful model organisms for the study of the mechanisms of salt tolerance in eukaryotic cells (77,78). Indeed, as the global problem of salinization of agricultural areas is rapidly increasing, these halotolerant and halophilic fungi now represent a source of target genes that can be used to convey increased salt tolerance to plants (79). Through the heterologous expression of *H. werneckii* genes in functional mutants of *S. cerevisiae* (80), its stress tolerance has been considerably improved. Initial studies on plants have shown that the insertion of the complete coding sequence of a 3'-phosphoadenosine-5'-phosphatase protein, ApHal2, from the halotolerant yeast *A. pullulans* into *Arabidopsis thaliana* improved halotolerance and drought tolerance of the transgenic plants. In a medium with an elevated salt concentration, the transformed plants were twice as likely to have roots in a higher length category in comparison with the wild type *Arabidopsis* and had 5 to 10 % larger leaf surface area under moderate and severe salt stress, respectively. This demonstrated that adaptations of extremotolerant fungi should be considered as a valuable resource for improving stress tolerance in plant breeding in the future (81).

Before studies of *H. werneckii* and other fungal model organisms began, the most studied eukaryotic microorganism regarding salt tolerance was *S. cerevisiae* (82), which is salt sensitive and cannot adapt to hypersaline conditions. Now a little more than a decade after the start of studies on halophilic and halotolerant fungi (12), we can conclude that these organisms represent a rich gene pool, as revealed with the genomic sequences of two fungal models, *H. werneckii* and *W. ichthyophaga*. Analyses of their genomes will therefore not only contribute

to our understanding of life in hypersaline environments, but should also identify further targets for improving the salt and osmotolerance of economically important plants and microorganisms.

Conclusions

The taxonomic and physiological characterization of halophilic fungi isolated from different salterns and hypersaline lakes on three continents have revealed their surprising diversity. New species have been described, and the primary natural ecological niche has been found for both rare species and for those that were previously known only as food contaminants. Most fungal species that inhabit these salterns have extremely halotolerant behaviour in comparison with their prokaryotic counterparts, whereby they do not require salt for viability, as they can grow and adjust to the whole salinity range, from freshwater to almost saturated NaCl solutions (34). The addition of fungi to other microbial communities in the salterns has improved our understanding of complex microbial processes in these natural hypersaline environments. Since evaporation and mineral precipitation are intimately linked to biological systems, microorganisms and their products can affect both the quality and quantity of salt (83). Although it has long been recognized that haloarchaea can be introduced into food *via* solar salt, causing the spoilage of heavily salted proteinaceous products (84,85), the contamination of food with fungi *via* this salt had previously been overlooked. Thus, it has only recently been shown that these fungi can also aid or harm salt production, and can contaminate salt that is used for food preservation (18,86,87). This contamination of food with potentially mycotoxigenic fungi that can synthesize toxins at high salinities (35,88,89) is not yet fully acknowledged, and it therefore requires further studies.

In recent years, another important ecological aspect in relation to fungi adapted to low a_w has been unraveled. Due to the general xerophilic nature of many representatives of saltern mycobiota, many fungal species that were initially detected in hypersaline environments were later, surprisingly, found in polythermal Arctic glaciers (18,28,30,34,90,91). Amongst the most surprising discoveries was perhaps the rich abundance of the halotolerant marine yeast *D. hansenii* in polar glaciers (90). Despite the extreme differences in physical conditions between glaciers and the solar salterns, the common critical parameter for many species found in both environments is the low a_w . This low a_w is due to the ice formation within glaciers and to the high salt concentrations within salterns. This special blend of properties is best suited to the black yeasts, which represent an integral part of these microbial communities, along with adapted populations of bacteria, archaea and a few other eukaryotic species.

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