

Mycosporines in Extremophilic Fungi—Novel Complementary Osmolytes?

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Environmental Context. *The occurrence of fungi in extreme environments, particularly in hypersaline water and in subglacial ice, is much higher than was previously assumed. When glacial ice melts as a result of calving or surface ablations, these organisms are released in the Arctic soil or sea and have a yet uninvestigated impact on the environment. Knowledge of the metabolites of these extremophilic fungi is important because they could provide signature molecules in the environment, but they can also contribute nutrients to the otherwise oligotrophic polar conditions. In the present work, we examine the osmotic behaviour of fungi grown under hypersaline conditions.*

Abstract. Fungi isolated from hypersaline waters and polar glacial ice were screened for the presence of mycosporines and mycosporine-like amino acids under non-saline and saline growth conditions. Two different mycosporines and three unidentified UV-absorbing compounds were detected by high performance liquid chromatography in fungal isolates from hypersaline waters and polar glacial ice. It was shown for the first time that the mycosporine–glutaminol–glucoside in halophilic and halotolerant black yeasts from salterns was higher on saline growth medium. This substance might act as a supplementary compatible solute in some extremophilic black yeasts exposed to saline growth conditions.

Keywords. halophilic/halotolerant — mycosporine-like amino acids (MAAs) — organic osmolyte — salt stress

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Introduction

Since the mid 1990s, discoveries of fungi growing in diverse extreme environments have broadened the study area of extremophilic microbiology, which has been traditionally dedicated to the study of prokaryotic microorganisms. Studies of extremophilic fungi have given insight into some of the eukaryotic adaptations to extreme growth conditions. The best-studied fungal extremophiles are halophilic and halotolerant fungi.^[1] Studies of fungal populations in natural hypersaline environments worldwide have revealed abundant and consistent occurrence of certain species.

The dominant fungi in eutrophic hypersaline waters are meristematic black yeasts, represented by halophilic *Horataea werneckii*, *Phaeothea triangularis*, *Trimmatostroma salinum*, halotolerant *Aureobasidium pullulans*,^[1] and different species of the genus *Cladosporium*, taxonomically and phylogenetically closely related to black yeasts.^[2,3] Besides, six different species of the teleomorph xerophilic genus *Eurotium* were repeatedly isolated,^[4] as well as the genus

Wallemia, a phylogenetic maverick in the *Basidiomycota*. The genus contains only three species, *W. muriae*, *W. sebi* and *W. ichthyophaga*, the latter being the most halophilic fungus known to date.^[5] Different species of *Aspergillus* and *Penicillium* and diverse non-melanised yeasts appear in hypersaline water of salterns less consistently.^[6]

Cold polar regions represent another extreme habitat in which fungi have been rarely investigated. An extraordinary aridity with a correspondingly low amount of biologically available water (also termed water activity, a_w), and occasional relatively high salt content is a common feature of these habitats.^[7] When comparing fungal species diversity from Arctic and hypersaline environments, we observed that most taxa of halotolerant and halophilic fungi from solar salterns worldwide were overlapping with the ones from a coastal Arctic environment.^[6]

Halophilic and halotolerant organisms thrive in environments with low a_w , because they can counterbalance the osmotic stress by accumulation of either high intracellular

salt concentrations or osmotically active compatible solutes. In addition to their function of maintaining the osmotic equilibrium across the cell membrane, compatible solutes are effective stabilisers of enzyme function, providing protection against temperature extremes, freeze–thaw treatment and even dryness.^[8] There is a great similarity between osmotic stress, caused by high concentrations of NaCl, and matric water stress, resulting from drought or binding of water into ice—the water activity of the habitat is low in both cases. Additionally, freezing and high salinity both lead to cellular dehydration and accumulation of free oxygen radicals, resulting in oxidative stress.^[9]

Microorganisms that adapt to moderate and high salt environments accumulate a diverse collection of organic osmolytes in response to water stress.^[10] Data on different halotolerant yeast species^[11,12] show that at high salinity, the maintenance of positive turgor pressure is mainly due to an increased production and accumulation of polyols. Commonly accumulated polyols include glycerol, erythritol, inositol, arabinitol, xylitol, and mannitol, with glycerol being by far the most important one.^[13,14]

Because both extracellular freezing and hypersaline stress lead to cellular dehydration, both can activate some common responses.^[15] These diverse stresses activate accumulation of compatible solutes, which can at the same time act as osmolytes and as cryoprotectants.^[16] Glycerol has been reported as the primary compatible solute in response to high osmolarity and low temperature in fungi.^[17]

In halophilic cyanobacteria, it was hypothesised that another group of compounds, the so-called mycosporine-like amino acids (MAAs), might also have an osmoregulative function.^[18] The diverse family of MAAs includes at least 19 different chemical species. They absorb maximally in the range 310–360 nm and contain preferentially an aminocyclohexenimine unit,^[19,20] whereas mycosporines have an aminocyclohexenone unit bound to an amino acid or amino alcohol group.^[19] In fungi, mycosporines were reported as water-soluble UV-absorbing (310–320 nm) compounds.^[20,21] Initially, they were discovered in fungal sporulating mycelia,^[22,23] but later on they were also found in carotenogenic basidiomycetous yeasts from Patagonia^[20] and in epilithic ascomycetous black meristematic fungi.^[24]

Considering the multiple roles of mycosporines and MAAs, especially the potential osmotic function of MAAs in cyanobacteria,^[18] we decided to screen a selected group of fungi for the presence of both groups of compounds. This screening study included experiments on the potential accumulation of mycosporines as a reaction to hypersaline conditions in fungal species inhabiting solely ice or hypersaline waters, as well as species found in both extreme environments. Salt-sensitive *Saccharomyces cerevisiae*^[25] was included in the study as a control.

Experimental

The studied fungi were identified previously as described elsewhere,^[26–28] and are maintained in a genetically stable way in the Culture Collection of the National Institute of Chemistry (MZKI, Ljubljana,

Slovenia) and in the Extremophilic Fungi (EXF) Culture Collection of the Department of Biology, Biotechnical Faculty, University of Ljubljana (Slovenia). Most penicillia are also preserved in the IBT fungal collection at the Centre for Microbial Biotechnology (BioCentrum-DTU), Lyngby, Denmark, whereas yeast-like strains are preserved in the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands.

Fungi were grown in liquid yeast nitrogen base (YNB) medium as described previously.^[29] Each fungal species (see Table 1) was grown in YNB medium without NaCl and with 10% w/w NaCl (or with 10% and 20% NaCl, or 5% and 10% NaCl in the case of *W. ichthyophaga* and *W. muriae* respectively) at 28°C and constant shaking (180 rpm) in the dark to mid-exponential growth phase. Then 100 µL of fungal cell suspension was inoculated in three parallels on cellophane overlaid on solid YNB growth media of the corresponding salinity. The cultures were incubated in normal daylight conditions at 24°C for 14 days on salt-free and for 30 days on NaCl-containing medium (owing to slower growth on the latter). The biomass was freeze-dried, the dry weight measured, and then samples were homogenised under liquid nitrogen using pestle and mortar.

Solid–liquid extraction procedures as described by Volkmann and Gorbushina^[30] were used to extract the UV-absorbing compounds from the samples.

Mycosporines and UV-absorbing substances were detected using a Waters 600 E high performance liquid chromatograph (HPLC; Waters, Milford, MA, USA) system equipped with a diode array detector (Waters 996), which was continuously scanning from $\lambda = 250$ to $\lambda = 400$ nm. A Lichrospher 100RP-18 column (5 µm, 4.6 × 220 mm, CS Chromatographie Service, Langerwehe, Germany) equipped with a guard pre-column containing the same material was used for separation. Twenty microlitres of the sample were injected onto the column using an autosampler system. The solution primarily used for the extraction (0.2% aqueous acetic acid + 0.5% methanol v/v) was used as a mobile phase. The flow rate was 0.7 mL min⁻¹. An extract of *Sarcinomyces petricola*, with known mycosporine composition,^[21,30] was used as an external standard for the retention times. Ultraviolet-absorbing mycosporines were identified by their UV-absorption maximum and retention time.^[30]

Results and Discussion

Two different mycosporines and three unidentified UV-absorbing compounds were detected in the extracts of the fungi screened. Mycosporines mycosporine–glutaminol–glucoside (Fig. 1, retention time (RT) 10 min, absorption maxima (λ_{\max}) 310 nm) and mycosporine–glutamicol–glucoside (Fig. 2, RT 14 min, λ_{\max} 310 nm) were identified on the basis of their RT and λ_{\max} (see Table 1). This identification was additionally confirmed by the previous characterisation studies with liquid chromatography–mass spectrometry (LC-MS).^[30] Three still unidentified UV-absorbing compounds

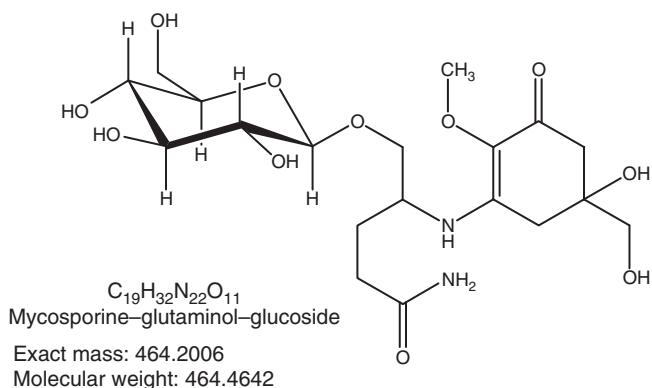


Fig. 1. Mycosporine–glutaminol–glucoside.

had absorption maxima exceeding 320 nm and thus probably belong to MAAs.

The patterns and the amounts of mycosporines synthesised differed among the species (see Table 1). Mycosporine–glutaminol–glucoside and mycosporine–glutamicol–glucoside were detected in halophilic black yeasts *Phaeothecha triangularis*, *Trimmatostroma salinum*, *Hortaea werneckii* and a halotolerant *Aureobasidium pullulans*, as well in a basidiomycetous yeast *Cryptococcus liquefaciens* (Fig. 3(a)). These mycosporines were previously unequivocally identified as metabolites of micro-colonial fungi inhabiting UV-exposed rocks in arid and semi-arid regions,^[21] as well as in some halotolerant and halophilic isolates, some of which were used in this ecophysiological screening experiment.^[30] Quantitative

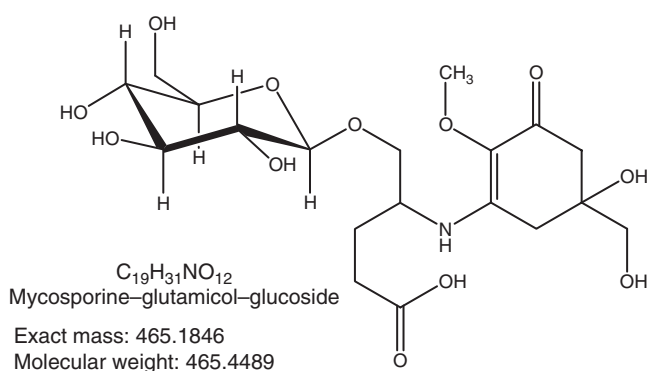


Fig. 2. Mycosporine–glutamicol–glucoside.

studies on mycosporine accumulation showed variability among the different species (Fig. 3). Within the first group of fungi, which contained halophilic black yeast species *H. werneckii*, *P. triangularis*, *T. salinum*, and a halotolerant *A. pullulans*, the amount of mycosporine–glutaminol–glucoside was higher on saline growth medium, whereas the amount of mycosporine–glutamicol–glucoside decreased. *Cryptococcus liquefaciens* also accumulated both mycosporine–glutamicol–glucoside and mycosporine–glutaminol–glucoside, but the amount of both mycosporines lowered when the cells were grown at 10% NaCl. The second group, which comprised *Aureobasidium* sp. isolated from a cold Arctic environment and isolates of *Cladosporium* sp. from a hypersaline environment, had only smaller amounts of mycosporine–glutamicol–glucoside. As in black yeasts, its amount decreased (Fig. 3(b)) in fungi grown on saline media. Smaller amounts of different UV-absorbing compounds were detected in *P. crustosum*, *S. cerevisiae*, *E. amstelodami* and *P. guilliermondii* (Fig. 3(c)). The amounts of these compounds in different species were not dependent on salt content of the medium.

We did not find any UV-absorbing compounds in *Rhodotorula mucilaginosa*, *Debaryomyces hansenii* or in *Walleimia* spp. (Table 1). We also confirmed the results of Libkind et al.,^[30] who did not detect either mycosporines or UV-absorbing compounds in the basidiomycetous yeast *Rhodospiridium babjevae*.

In the present report, out of 18 fungal strains from natural hypersaline and Arctic habitats, eight were able to

Table 1. Fungal species, their origin, and their mycosporine and mycosporine-like amino acids content

Species	Strain No.	Origin	Myc–Gln–Glu ^D (Rt 10 min)	Myc–Glc–Glu ^E (Rt 14 min)	UVas-325 ^F (Rt 5 min, λ_{\max} 325 nm)	UVas-335 ^F (Rt 5 min, λ_{\max} 335 nm)	UVas-329 ^F (Rt 6 min, λ_{\max} 329 nm)
<i>Phaeothecha triangularis</i> ^A	EXF-206	Salterns	+	+	–	–	–
<i>Trimmatostroma salinum</i> ^A	EXF-295	Salterns	+	+	–	–	–
<i>Hortaea werneckii</i> ^A	MZKI B-736	Salterns	+	+	–	–	–
<i>Aureobasidium pullulans</i> ^A	EXF-150	Salterns	+	+	–	–	–
<i>Cryptococcus liquefaciens</i> ^B	MZKI K428	Arctic	+	+	–	–	–
<i>Aureobasidium</i> sp. ^A	EXF-1940	Arctic	+	–	–	–	–
<i>Cladosporium cladosporioides</i> ^A	EXF-381	Salterns ^C	+	–	–	–	–
<i>Cladosporium sphaerospermum</i> ^A	EXF-385	Salterns ^C	(+)	–	–	–	–
<i>Penicillium crustosum</i> ^A	EXF-1046	Arctic ^C	–	–	–	+	+
<i>Saccharomyces cerevisiae</i> ^A	MZKI K86	Wild-type control	–	–	+	–	(+)
<i>Eurotium amstelodami</i> ^A	EXF-66	Salterns ^C	–	–	(+)	–	–
<i>Pichia guilliermondii</i> ^A	EXF-518	Salterns ^C	–	–	(+)	–	–
<i>Rhodospiridium babjevae</i> ^B	EXF-513	Salterns	–	–	–	–	–
<i>Rhodotorula mucilaginosa</i> ^B	EXF-1630	Arctic ^C	–	–	–	–	–
<i>Debaryomyces hansenii</i> ^A	EXF-589	Salterns ^C	–	–	–	–	–
<i>Walleimia ichthyophaga</i> ^B	EXF-994	Salterns	–	–	–	–	–
<i>Walleimia muriae</i> ^{B,N}	EXF-951	Salterns	–	–	–	–	–
<i>Walleimia sebi</i> ^{B,N}	EXF-958	Sunflower seed	–	–	–	–	–

MZKI, Culture Collection of the National Institute of Chemistry, Ljubljana, Slovenia; EXF, Extremophilic Fungi (EXF) Culture Collection of the Department of Biology, Biotechnical Faculty, University of Ljubljana, Slovenia; ^AAscomycetes; ^BBasidiomycetes; ^Nneotype strain; ^Cspecies isolated from both extreme environments; ^Dmycosporine–glutaminol–glucoside; ^Emycosporine–glutamicol–glucoside; ^FUV-absorbing substances named by their absorption maxima; + detected; (+) detected in one or two cultures out of three; – not detected; Rt, retention time.

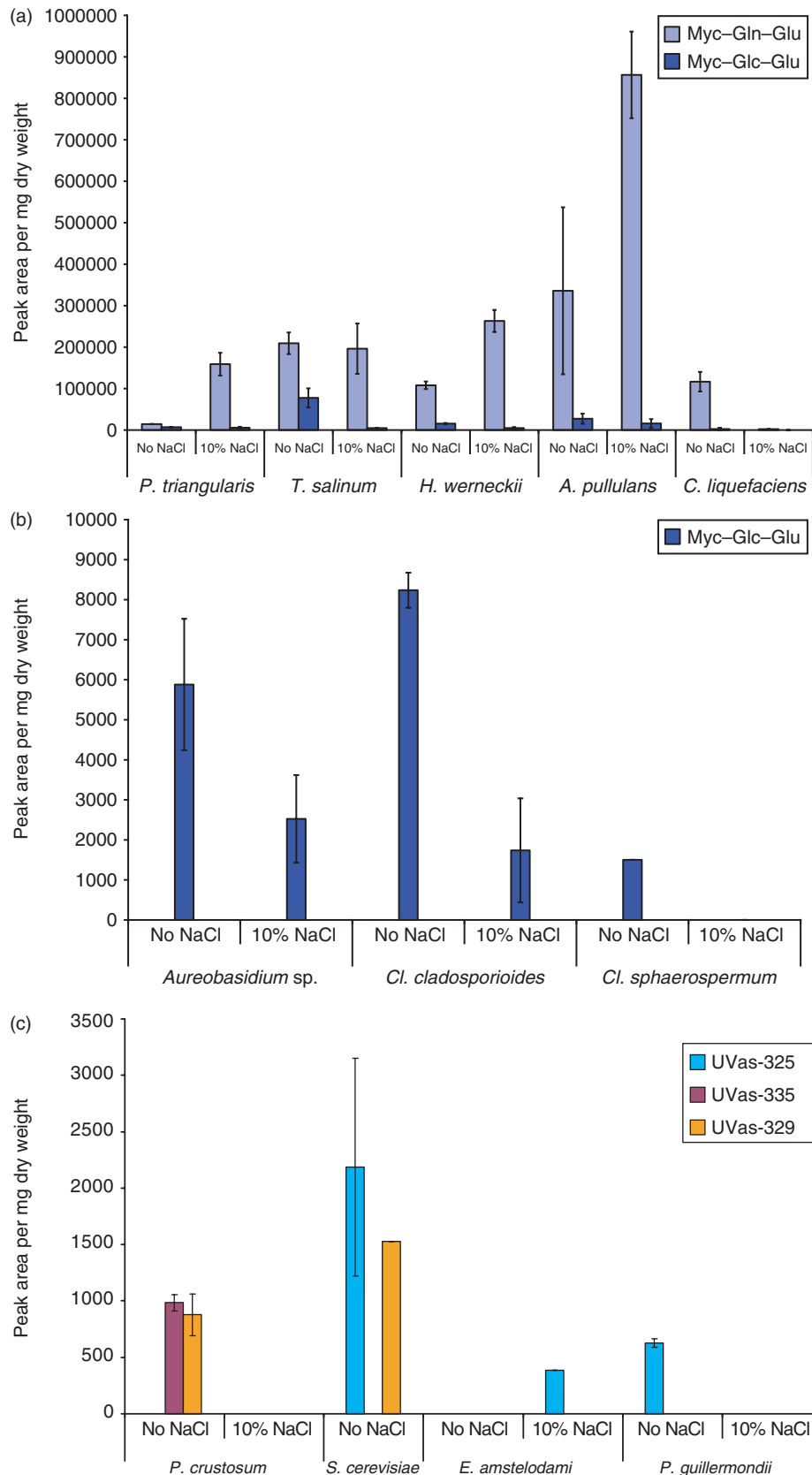


Fig. 3. Amounts of mycosporines and UV-absorbing substances detected in tested fungal species by high performance liquid chromatography. (a) Group of black yeasts and *Cryptococcus liquefaciens* accumulated mycosporine–glutaminol–glucoside (Myc–Gln–Glu) and mycosporine–glutamicol–glucoside (Myc–Glc–Glu). (b) Group 2 accumulated only Myc–Glc–Glu. (c) This group accumulated only UV-absorbing substances. The amounts are expressed in relative values and normalised to dry weight. The values are the average of measurements of three parallel samples \pm standard deviation, except in *Cladosporium sphaerospermum* without NaCl, *Saccharomyces cerevisiae*, *Eurotium amstelodami* at 10% NaCl, and *Pichia guillermondii* without NaCl, where the peak area values below the detection limit were not included in the average values shown.

synthesise mycosporines and four contained yet unidentified UV-absorbing compounds, possibly MAAs.

These results indicate that some mycosporines might act as supplementary compatible solutes in some extremophilic fungi exposed to variations in a_w or to hypersaline conditions. The group of halophilic and halotolerant black yeasts accumulated more mycosporine–glutaminol–glucoside in response to osmotic stress, with the exception of *T. salinum*. Although the group is small in number, these halophilic species have the dominant ecological role among fungi inhabiting hypersaline environments. The significant salt-correlated increase in mycosporine–glutaminol–glucoside content might support an idea that mycosporines help these stress-tolerant fungal species to better tolerate the osmotic challenge. Similar osmotic function was already proposed for MAA in halophilic cyanobacteria,^[18] but in the cyanobacterium *Chlorogloeopsis* sp., the correlation between MAA content and osmotic stress was found to be insignificant when the amount of MAA was compared with intracellular concentrations of sugar osmolytes.^[31]

Mycosporines in fungi were initially reported as being related to spores and other reproductive or survival structures, such as fruit bodies or sclerotia, and recently in yeasts as UV photoprotective agents. Based on chemical considerations, fungal oxo-mycosporines could also be redox-active (i.e. have antioxidant activity), and in certain cases, they have evolutionarily assumed even additional functions.^[32] Initial reports on mycosporines indicated that their presence was restricted to only a few fungal species, whereas recent results of Libkind et al.^[33] suggest that mycosporinogenesis is a consistent character related to certain phylogenetic lineages, suggesting that this trait was already present in the group's common ancestor. Fungal groups now incapable of mycosporine production may have lost this function during evolution. The presence of mycosporines in pigmented yeasts and yeast-like fungi,^[24,33] already protected by pigments from harmful UV irradiation, could be related to other environmental factors of evolutionary significance. The strains in our study cultivated on media without salt produced only light-induced mycosporines, whereas the amount of mycosporine–glutaminol–glucoside was clearly salt-dependent. We think that the amount and also the type of mycosporines could be stress-dependent in general; therefore, one needs to be careful in choosing growth conditions before using mycosporines as additional taxonomic markers as proposed by Gorbushina et al.^[24] and Libkind et al.^[33]

This suggests that halophily,^[34] as well as the ability to produce mycosporines or MAAs, is either an evolutionarily ancient trait (a plesiomorph) within these orders, or that different species or genera had adapted to extreme environmental conditions independently. If its origin is evolutionarily old, there may be a general constitutional and physiological cellular mechanism underlying such behaviour, suggesting that a general type of stress response underlies diverse extremophilic types of ecology.

Further studies are needed to verify whether mycosporine–glutaminol–glucoside might indeed play an osmoprotective role in fungi living in extreme environments. Also,

investigations of mycosporines' other potential roles, helpful in overcoming various other hostile conditions (such as growth at high salinity and low temperatures) are lacking.

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