A First Record of Obligate Halophilic *Aspergilli* from the Dead Sea

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Abstract The isolation of obligate halophilic aspergilli from the Dead Sea and the range of salt tolerance of halophilic fungi isolated, are reported here for the first time. The mycobiota of the Dead Sea isolated in this study, was dominated by *Aspergillus* and *Penicillium* species; *Cladosporium* were found in lesser numbers. All three genera were obtained from the water sample; however, *Aspergillus* was the only genus obtained from the sediment. There was significant difference in growth of each isolate at different salt concentrations and intraspecies analysis revealed dissimilarity in response of strains to different salt concentrations in the growth medium. The isolates were euryhaline, with halotolerance up to 20–25% solar salt, *Aspergillus* and *Penicillium* species showing a higher level of halotolerance, as compared to that of *Cladosporium*. Halophilic fungi were found in greater numbers in the sediment sample as compared to that in the water sample. *Penicillium* and *Cladosporium* species were exclusively facultative halophiles, while some species of *Aspergillus* were facultative halophiles. All the obligate halophiles isolated, belonged to the genus *Aspergillus* and were identified as *A. penicilliioides* and *A. unguis*, the latter being a first record of the species from the Dead Sea.

Keywords Dead Sea · Obligate halophile · *Aspergillus* · *Penicillium* · *Cladosporium*

Introduction

The hypersaline environment contains salt concentrations in excess of sea water [1]. Hypersaline conditions drastically decrease the biodiversity by exerting a strong selective pressure, resulting in the development of halophilic and halotolerant microbes [2], the study of which has drawn much interest [3]. Fungi are known to adapt to certain extreme environments, but isolation and identification of fungi from these habitats have been frequently hindered due to their slow growth and low ability to compete [4].

The Dead Sea, an extremely hypersaline environment, has high concentrations of about 340‰ total dissolved salts, with levels of divalent ions like magnesium and calcium being higher than that of monovalent ions. This unique ionic composition makes it hostile to microorganisms and could be responsible for the paucity in the diversity of the Dead Sea biota [5, 6]. Microbes that inhabit high-salt environments are adapted to high levels of ions, as well as to low a_w , and are described as halophilic or halotolerant, while the term xerophilic or xerotolerant is restricted to describing those organisms growing at a_w values imposed by *other than* inorganic ions [7].

A number of filamentous fungi have been isolated from the Dead Sea [3, 5, 6]; however, only the species of *Gymnascella marismortui* has been reported as a true halophile, having an absolute requirement of salt for growth [5, 8].

The present study was aimed at investigating the existence of obligate halophilic fungi in the Dead Sea, and records the isolation of obligate halophilic aspergilli for the first time. It also illustrates the range of salt tolerance of halophilic fungi, differentiating these as obligate or facultative halophiles, an aspect which has been insufficiently studied hitherto.

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Materials and Methods

Sample Collection

Dead Sea water (DSw) and sediment (DSs) samples were aseptically collected at Kalya beach (31°45′50.62″N and 35°30′13.10″E) at about 0.5 m depth and 3–4 m from the shore.

Salinity and pH Measurement

Salinity was measured with an Atago refractometer; pH was recorded by Cyberscan pH meter. DSs sample was suspended in deionised water (1:5 w/v), centrifuged at 3000 rpm, 10 min (Remi centrifuge), to remove particulate matter, before analysis.

Isolation and Identification of Fungi

DSw and a suspension of DSs were each plated on 10 plates of Czapek-Dox Agar + 20% solar salt (20%S-CzA) and on CzA + 10% solar salt (10%S-CzA) plate, then incubated at 30°C for 30 days.

All isolates obtained from the water sample were picked; those obtained from sediment, being in greater numbers, were selected based on apparent dissimilarity in cultural characteristics. Purified isolates were identified on basis of colony and micromorphology characteristics with reference to identification keys [9–11].

The identification of selected obligate halophilic isolates was confirmed by ITS rDNA sequence analysis (Merck-GeNei Services), using consensus primers for 18S rRNA, ITS1, 5.8S rRNA, ITS2 and 28S rRNA gene fragment, using PCR conditions of one cycle of denaturation at 94°C (5 min), followed by 35 cycles of denaturation at 94°C (1 min), annealing at 55°C (45 s) and extension at 72°C (1.30 min), then maintained at 72°C (10 min). Sequence data was analyzed to find the closest homologs; GenBank accession numbers were obtained. Sequence similarities were obtained using NCBI BLAST. Alignment and phylogenetic tree were constructed in Clustal X version 2 and the NJ distance method.

Determination of Halotolerance

Spore suspensions of isolates were prepared in 2% saline containing Tween 80 (0.05% final concentration), and spot-inoculated on CzA containing solar salt (0–30%). These were subcultured by inoculating 10^3 spores in triplicate on plates containing corresponding salt concentrations. Growth was recorded in terms of colony diameter after 7 days incubation, or after 15 days for those showing delayed growth.

Statistical Analysis

The correlation of viable counts with salinity of the sample, or, with salinity of isolation media was performed by the Pearson correlation test. Fungal diversity of water or of sediment, each on 10%S-CzA and on 20%S-CzA, was analyzed by Shannon–Weaver index (H). Statistically significant difference ($P < 0.05$) in the effect of different salt concentrations on growth of all isolates, as well as in growth within the species, was analysed by two-way ANOVA.

Results

Salinity and pH

The DSw sample had a salinity of 370‰ and pH of 5.65; DSs showed 450‰ salinity, pH 5.3.

Density and Diversity of Fungal Isolates

The fungal count of DSw was 0.2 cfu ml^{-1} on 20% S-CzA and 0.1 cfu ml^{-1} on 10% S-CzA; DSs yielded 91.1 cfu g^{-1} on 20% S-CzA and 8.9 cfu g^{-1} on 10% S-CzA. The density of fungal isolates showed a positive correlation with salinity of the sample ($r = 1$) and with salt concentration used in the isolation medium ($r = 1$).

Twenty-five isolates were obtained from DSw: twenty-one were isolated on 20% S-CzA, of which eight were identified as *Aspergillus*, twelve as *Penicillium* and one with sterile mycelia; four isolates were obtained on 10% S-CzA, identified as *Cladosporium*. DSs was dominated exclusively by aspergilli; thirty from the total of 414 obtained on 20% S-CzA and all four obtained on 10% S-CzA were picked for further study. The identification of the isolates is shown in Table 1. On 20% S-CzA, DSw showed seven species ($H = 0.72$), DSs yielded two species ($H = 0.15$); on 10% S-CzA, DSw and DSs each showed only one species ($H = 0$).

Halotolerance

Figure 1 shows the halotolerance of an individual isolate, of isolates within a species with statistically significant difference, or of an isolate as indicative of a group having similarity. The halophilic nature of each isolate is indicated in Table 1.

Isolates from Water Sample

Five of the eight aspergilli—DSw1, DSw5, DSw6, DSw17 and DSw22, did not grow in absence of salt and were

Table 1 Fungi from Dead Sea water and sediment samples isolated on Czapek-Dox Agar supplemented with 10 or 20% solar salt

Isolate	Sample	Solar salt added to CzA (%)	Isolate number	Halophilic nature
<i>A. penicillioides</i>	Water	20	DSw1, DSw5, DSw6, DSw17, DSw22	Obligate
<i>A. unguis</i>	Water	20	DSw12	Obligate
<i>A. restrictus</i>	Water	20	DSw14	Facultative
<i>A. flavus</i> var. <i>columnaris</i>	Water	20	DSw9	Facultative
<i>P. corylophilum</i>	Water	20	DSw4, DSw8, DSw10, DSw11, DSw13, DSw15, DSw18, DSw19, DSw20.	Facultative
<i>P. steckii</i>	Water	20	DSw7, DSw21	Facultative
<i>P. waksmanii</i>	Water	20	DSw16	Facultative
<i>C. cladosporioides</i>	Water	10	DSw23, DSw24, DSw25, DSw26	Facultative
Sterile mycelia	Water	20	DSw2	Obligate
<i>A. penicillioides</i>	Sediment	20	DSs28, DSs29, DSs30, DSs31, DSs32, DSs33, DSs34, DSs35, DSs36, DSs37, DSs38, DSs39, DSs40, DSs41, DSs42, DSs43, DSs44, DSs45, DSs46, DSs49, DSs53, DSs54, DSs56	Obligate
			DSs47, DSs48, DSs51, DSs55	Facultative
<i>A. versicolor</i>	Sediment	20	DSs50, DSs52, DSs57	Facultative
		10	DSs58, DSs61	Facultative
			DSs59, DSs60,	Halotolerant

obligate halophiles, showing identical cultural and morphological characteristics; the ITS sequence analysis of one from this group, DSw22, confirmed it to be *A. penicillioides*. Similarly, DSw12, also an obligate halophile and DSw14 which showed delayed growth in absence of added salt, were identified by ITS sequence analysis as *A. unguis* and *A. restrictus*, respectively. The GenBank accession numbers were HQ702381 for DSw12, HQ702382 for DSw14, HQ702383 for DSw22. The phylogenetic relationship of these isolates is shown in Fig. 2. The other isolates grew in absence of added salt, but showed enhanced growth at increased levels of salt and were termed as facultative halophiles; these were identified as *A. flavus* var. *columnaris*, *Penicillium* species: *P. corylophilum*, *P. steckii*, *P. waksmanii* and *Cladosporium cladosporioides*. The sterile fungus was an obligate halophile.

The six obligate halophilic aspergilli: five *A. penicillioides* and *A. unguis* grew in presence of 2 to 20–25% salt, with maximal growth at 10 and 10–15% salt, respectively; the facultative halophiles *A. restrictus* and *A. flavus* var. *columnaris*, grew optimally at 5% salt, with tolerance of 20% salt during growth.

The obligate halophilic sterile fungus grew in presence of 2–15% salt and maximally at 10% salt.

The facultative halophilic penicilli cultures all required 2–5% salt for optimal growth. *P. corylophilum* had maximum salt tolerance of 15% (two isolates), 20% (six isolates), or 25% (one isolate); tolerance levels of *P. steckii* and *P. waksmanii* were 20–25% salt. The *Cladosporium* isolates were also facultative halophiles, which grew

optimally with 5% salt and showed tolerance of 15–20% salt.

Significant difference ($P < 0.05$) was obtained in growth of each isolate at different salt concentrations, as well as in growth amongst the strains of *A. penicillioides*, *C. cladosporioides* and *P. corylophilum*; however, the strains of *P. steckii* were similar.

Isolates from Sediment Sample

Twenty-three of the isolates from DSs isolated on 20% S-CzA, did not grow in absence of salt and were obligate halophiles, identified as *A. penicillioides*; DSs35 and DSs40, one each from amongst those showing delayed growth at salt concentrations of 2 and 5% salt, respectively, were confirmed by gene sequence analysis as *A. penicillioides*; the GenBank accession numbers were HQ702384 for DSw35 and HQ702385 for DSw40. Figure 2 indicates the phylogenetic relationship of these isolates. Four other isolates which showed delayed growth in absence of salt were also identified as *A. penicillioides* and three facultative halophiles were identified as *A. versicolor*. The four isolates obtained on 10% S-CzA plates were identified as *A. versicolor*.

The twenty-three obligate halophilic *A. penicillioides* were grouped on the basis of the minimum requirement of 2 or 5% salt for growth, fifteen and eight isolates, respectively, with some of these showing delayed growth at the minimum salt requirement. Two isolates with slow growth at 2% salt, grew optimally with 5% salt, while the other

Fig. 1 Salt tolerance curves of the isolates from Dead Sea water and sediment samples as recorded after 7 days incubation; unconnected *open symbols* indicate delayed growth at respective salt concentrations, after 15 days incubation

Fig. 2 Phylogenetic tree obtained from the alignment of ITS region of rDNA of *Aspergillus* species; isolates obtained from the present study are indicated in **bold**, along with the GenBank accession numbers

isolates grew optimally with 10% salt; all tolerated 20% salt concentration, with some of these growing even at 25% salt within 15 days.

Three of the facultative halophilic *A. penicillioides* grew at 20% salt, with optimum growth of one at 5% and two at 10% salt; the fourth isolate grew at 25% salt within 15 days, and optimally at 10% salt.

The facultative *A. versicolor*, three isolated on 20% S-CzA and one on 10% S-CzA, all grew at a range of 0–20% salt with maximal growth obtained in presence of 2, 5 or 10% salt. Two other isolates picked from 10% S-CzA, were halotolerant, with optimal growth in absence of added salt, and decreased growth in presence of increased levels of salt.

Significant difference ($P < 0.05$) was obtained for the growth of isolates at different salt concentrations. Analysis of growth of *A. penicillioides* at different salt concentration revealed that those strains that required a minimum of 2% salt for growth were dissimilar, while those that required a minimum of 5% salt for growth, were similar. There was also similarity among strains of the facultative halophilic *A. penicillioides* and among that of *A. versicolor*, all isolated on 20% S-CzA; however, there was dissimilarity between strains of *A. versicolor* isolated on 10% S-CzA.

Discussion

The Dead Sea is a hypersaline environment with a unique ecology, its salinity being higher than the recorded 275‰ of the Great Salt Lake, Utah [1, 8]; the study of its microbiota has attracted much interest in recent decades. The acidic pH of the Dead Sea, as recorded herein and confirmed in earlier reports, is unlike other neutral or alkaline hypersaline lakes [12] and would be conducive to fungal life.

The fungi obtained in the present work could be native as well as of terrestrial source, brought by the influx of water from the Jordan River, or through water movement at the shore line, as well as by airborne contamination and human activity. Microbes may be present in the dormant state, able to return to the physiologically active state under favourable conditions [13]; mycelial forms may develop only during the rare episodes wherein the salinity of the upper water layers is reduced as a result of inflow of freshwater. The high concentration of salt used for fungal isolation in this study, and the avoidance of a rich/more easily assimilable carbon medium, would not only have aided the selection of halophiles, but also served to inhibit the growth of any faster-growing terrestrial fungi.

Aspergillus and *Penicillium* were the principal genera obtained with the methodology used in this study. Diversity studies have revealed that at low water potential the active

mycota is dominated by species of *Aspergillus* and *Penicillium*, which are thus numerically the most common taxa [2], as observed in studies on Mono Lake [14], salterns of Slovenia [15], Arctic [16] and Cabo Rojo, [17], and our own studies on local salterns (unpublished data). Amongst the species obtained in this study, the isolation of *A. unguis* is a first report, while *A. penicillioides*, *A. restrictus*, *A. versicolor*, *C. cladosporioides*, *P. steckii* and *P. corylophilum* have been recorded in earlier work on the Dead Sea mycobiota [2, 3, 18, 19], *A. versicolor* and *C. cladosporioides* having been reported as indigenous species [18]. The isolation of these fungi in the summer months corroborates the report of Kis-Papo et al. [18].

It was observed that while almost all of the fungi from different genera were halophilic in nature, obligate halophiles were found only in the genus *Aspergillus*, namely *A. penicillioides* and *A. unguis*, which could be indigenous fungi; these species add to the recorded strains of the obligate halophilic *Gymnascella marismortui* [5]. *A. versicolor* and *A. flavus* var. *columnaris*, *Penicillium* and *Cladosporium* species were facultative halophiles. Although some of these species have been reported earlier, their halophilic nature as obligate or facultative halophiles, or halotolerant, had not been defined.

It was interesting to note that the facultative halophiles *Penicillium* and *Cladosporium* were found exclusively in the water, while the sediment yielded only *Aspergillus* species, mainly the obligate halophilic *A. penicillioides* and, in lesser numbers, the facultative halophiles *A. penicillioides* and *A. versicolor*. The facultative halophilic *A. flavus* var. *columnaris* and *A. restrictus*, were isolated only from the water, while *A. versicolor* was obtained only from the sediment. Notably, *Cladosporium* species were isolated only on medium containing 10% salt but not in presence of 20% salt, indicating that the genus as a whole is not as halotolerant as the genera of *Aspergillus* and *Penicillium*. Furthermore, *A. penicillioides* was present in high density and formed the principal fungi in the sediment sample, where, the a_w would be low due to the higher matrix potential and salinity [13].

The precipitation of halites from the water column due to evaporation [12] could be responsible for the higher salinity of the sediment as compared to that of the water; this could be a reason for obligate halophiles to survive more readily and in greater numbers, in the sediment, while the facultative halophiles were found more in the water column. Only those organisms able to withstand these conditions can thrive [13], which could explain the lower diversity obtained in the sediment.

The obligate halophiles isolated were observed to grow slower, yielding smaller colonies than the facultative halophiles and halotolerant fungi. Marine fungi are noted to grow very slowly on artificial media as compared to

terrestrial fungi [20]; the reduced growth rate of salt adapted cultures may be related to the increase in energy demands under stress, wherein cells may utilize energy for compartmentalization or exclusion of Na⁺ ion, direct synthesis of compatible osmolytes and synthesis of proteins conferring salt tolerance [21].

The isolates were obtained in greater numbers on isolation medium containing 20% salt, as compared to that obtained on medium with 10% salt, and showed an extreme halotolerance of 20–25% salt; however, no extreme halophile was obtained, the isolates being mainly moderate halophiles. The isolates were euryhaline in nature, growing over a wide range of salt concentrations.

In the present study, it was observed that while almost all of the fungi from different genera were halophilic in nature, obligate halophiles were found only in the genus *Aspergillus*: *A. penicillioides* and *A. unguis*, which could be indigenous mycobiota; these species add to the known strains of *Gymnascella marismortui*. [5]. *A. versicolor* and *A. flavus* var. *columnaris*, *Penicillium* and *Cladosporium* species were facultative halophiles. Although some of these species have been reported earlier, their halophilic nature as obligate or facultative halophiles, or halotolerant, had not been defined.

This is a first description of aspergilli species as obligate halophiles and those of the genera *Penicillium* and *Cladosporium* as facultative halophiles, establishing all the isolates as euryhaline in nature; it is also a first report of obligate halophilic *A. penicillioides* and *A. unguis* from the Dead Sea.

References

1. DasSarma S, Arora P (2002) Halophiles. In: Encyclopedia of life sciences, vol 8. Nature Publishing Group, London, pp 458–466
2. Das S, Lyla PS, Khan SA (2006) Marine microbial diversity and ecology: importance and future perspectives. Curr Sci 90:1325–1335
3. Grishkan I, Nevo E, Wasser SP (2003) Soil micromycete diversity in the hypersaline Dead Sea coastal area, Israel. Mycol Prog 2:19–28
4. Gunde-Cimerman N, Plemenitaš A (2006) Ecology and molecular adaptation of the halophilic black yeast *Hortaea werneckii*. Rev Environ Sci Biotechnol 5:323–331
5. Buchalo AS, Nevo E, Wasser SP, Oren A, Molitoris HP (1998) Fungal life in the extremely hypersaline water of the Dead Sea: first records. Proc R Soc Lond 265:1461–1465
6. Kis-Papo T, Oren A, Wasser SP, Nevo E (2003) Survival of filamentous fungi in hypersaline Dead Sea water. Microbial Ecol 45:183–190
7. Grant WD (2004) Life at low water activity. Phil Trans R Soc Lond B 359:1249–1267
8. Kushner DJ (1978) Life in high salt and solute concentrations. In: Kushner DJ (ed) Microbial life in extreme environments. Academic Press, London, pp 317–368
9. Domsch KH, Gams W, Anderson TH (1980) Compendium of soil fungi, vol 1. IHW-Verlag, Eching
10. Ellis MB (1971) Dematiaceous hyphomycetes. Commonwealth Mycological Institute, Kew, Surrey, England
11. Raper KB, Fennell DI (1965) The genus *Aspergillus*. Williams and Wilkins Co, Baltimore
12. Oren A (2003) Physical and chemical limnology of the Dead Sea. In: Nevo E, Oren A, Wasser SP (eds) Fungal life in the Dead Sea. Gantner Verlag, Ruggel, pp 45–67
13. Kunte JH, Truper HG, Stan-Lotter S (2002) Halophilic microorganisms. In: Horneck G, Baumstark C (eds) Astrobiology: the quest for the conditions of life. Springer, Berlin, pp 185–199
14. Steiman R, Ford L, Ducros V, Lafond JL, Guiraud P (2004) First survey of fungi in hypersaline soil and water of Mono Lake area (California). Antonie van Leeuwenhoek 85:69–83
15. Gunde-Cimerman N, Cerovac S, Zalar P (2001) Biodiversity of filamentous fungi in the salt pans Secovlje. Acta Biol Slov 44:25–30
16. Gunde-Cimerman N, Lorena B, Sonjak S, Turk M, Uršič V, Zalar P, Plemenitaš A (2005) Halotolerant and halophilic fungi from coastal environments in the Arctics. In: Gunde-Cimerman N, Oren A, Plemenitaš A (eds) Adaptation to life at high salt concentrations in Archaea bacteria and Eukarya. Springer, The Netherlands, pp 397–423
17. Cantrell SA, Casillas-Martinez L, Molina M (2006) Characterization of fungi from hypersaline environments of solar salterns using morphological and molecular techniques. Mycol Res 110:962–970
18. Kis-Papo T, Grishkan I, Gunde-Cimerman N, Oren A, Wasser SP, Nevo E (2003) Spatiotemporal patterns of filamentous fungi in the hypersaline Dead Sea. In: Nevo E, Oren A, Wasser SP (eds) Fungal life in the Dead Sea. Gantner Verlag, Ruggel, pp 271–292
19. Wasser SP, Grishkan I, Kis-Papo T, Buchalo AS, Paul AV, Gunde-Cimerman N, Zalar P, Nevo E (2003) Species diversity of the Dead Sea fungi. In: Nevo E, Oren A, Wasser SP (eds) Fungal life in the Dead Sea. Gantner Verlag, Ruggel, pp 203–270
20. Ravishankar JP, Suryanarayanan TS, Muruganandam (2006) Strategies for osmoregulation in the marine fungus *Cirrenalia pygmaea* Khol. (Hyphomycetes). Indian J Mar Sci 34:351–358
21. Redkar RJ, Lemke PA, Singh NK (1996) Altered gene expression in *Aspergillus nidulans* in response to salt stress. Mycologia 88:256–263