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Hydrophilins in the filamentous fungus *Neosartorya fischeri* (*Aspergillus fischeri*) have protective activity against several types of microbial water stress

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Abstract

Hydrophilins are proteins that occur in all domains of life and protect cells and organisms against drought and other stresses. They include most of the late embryogenesis abundant (LEA) proteins and the heat shock protein (HSP) Hsp12. Here, the role of a predicted LEA-like protein (LeamA) and two Hsp12 proteins (Hsp12A and Hsp12B) of *Neosartorya fischeri* was studied. This filamentous fungus forms ascospores that belong to the most stress-resistant eukaryotic cells described to date. Heterologous expression of LeamA, Hsp12A and Hsp12B resulted in increased tolerance against salt and osmotic stress in *Escherichia coli*. These proteins were also shown to protect lactate dehydrogenase against dry heat and freeze–thaw cycles *in vitro*. Deletion of *leamA* caused diminished viability of sexual ascospores after drought and heat. This is the first report on functionality of Hsp12 and putative LeamA proteins derived from filamentous fungi, and their possible role in *N. fischeri* ascospore resistance against desiccation, high temperature and osmotic stress is discussed.

Introduction

Adverse conditions such as freezing, drought and variations in osmotic potential confront organisms with a limitation of cellular water, affecting the conformation of proteins and membranes (Hoekstra *et al.*, 2001). Desiccation-tolerant organisms possess protective mechanisms including intracellular accumulation of compatible solutes and protective proteins. Compatible solutes consist of sugars, polyols, amino acids and derivatives thereof. They stabilize cellular structures and restore the osmotic balance without interfering with the functioning of biomolecules (Leslie *et al.*, 1995; Shen *et al.*, 1999; Billi *et al.*, 2000). Protective proteins bind to and structurally stabilize other proteins, membranes and RNA (Warner *et al.*, 2004).

Hydrophilins are an important class of protective proteins that include the late embryogenesis abundant (LEA) proteins (Battaglia *et al.*, 2008) and a heat shock protein (HSP) called Hsp12 (Praekelt and Meacock, 1990). They are characterized by a high glycine content (> 6%) and a high hydrophilicity index (> 1) as stated by Garay-Arroyo and colleagues (2000). Initially, LEA proteins are described during late stages of embryo development in plant seeds (Dure *et al.*, 1981), but now recognized in all kingdoms of life (Honjoh *et al.*, 1995; Garay-Arroyo *et al.*, 2000; Katinka *et al.*, 2001; Browne *et al.*, 2002; Tanaka *et al.*, 2004; Kikawada *et al.*, 2006; Campos *et al.*, 2013). Sequence comparisons by computer programs suggest that hydrophilins fold into amphipathic α-helices (Dure *et al.*, 1989), but the proteins are intrinsically disordered in solution (Dyson and Wright, 2005; Mouillon *et al.*, 2006). This is illustrated by the finding that a group 3 LEA protein (PsLEAm) in the nematode *Aphelenchus avenea* showed upon dehydration a dramatic, but reversible shift from natively unfolded towards the presence of α-helices (Goyal *et al.*, 2003). By folding into amphipathic α-helices, hydrophilins may stabilize phospholipids and membranes (Sales *et al.*, 2000; Kalemba and Pukacka, 2007; Tolleter *et al.*, 2007; Welker *et al.*, 2010; Popova *et al.*, 2011) and might prevent protein aggregation due to water stress (Goyal *et al.*, 2005). Further, these proteins strengthen the glassy matrix of trehalose (Shimizu *et al.*, 2010) or sucrose (Wolkers *et al.*, 2001) to a higher extent.

Hsp12 of *Saccharomyces cerevisiae* is a hydrophilin and as such structurally and functionally different from all other known HSPs. Deletion of *hsp12* has no effect on growth and viability of *S. cerevisiae* at low and high temperatures (Praekelt and Meacock, 1990). This is explained by overproduction of trehalose as a compensatory mechanism as illustrated by the fact that overexpression of *hsp12* in a trehalose-6-phosphate synthase null mutant does increase freeze and heat

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resistance (Pacheco *et al*., 2009). Several studies provide evidence that Hsp12 folds into a structure containing α-helices only in the presence of lipids (Welker *et al*., 2010; Herbert *et al*., 2012).

We here present a first report on the functionality of the hydrophilins Hsp12 and a putative LeamA protein in ascospore resistance against desiccation, high temperature and osmotic stress in the fungus *Neosartorya fischeri*. This fungus causes spoilage of food products after pasteurization as the extreme stress-resistant sexual spores it forms are resistant to temperatures above 80°C (Beuchat, 1986).

Results

In silico *analysis of LeamA, Hsp12A and Hsp12B*

The genome of *N. fischeri* contains one gene encoding a predicted LEA-like protein and two genes encoding Hsp12 proteins. The open reading frame of the predicted LEA gene, *leamA* (NFIA_065760; NCBI-geneID: 4589955), encodes a highly hydrophilic protein of 140 amino acid residues and a glycine content of 9.3% (Fig. 1). The C-terminal part of LeamA contains three intact and one truncated 11-mer motif repeat (Fig. 1C), which is a characteristic of PsLEAms (Dure, 1993a,b). Comparison of the 11-mer motif of LeamA of *N. fischeri* with homologues of the related ascomycetes *Aspergillus fumigatus*, *Aspergillus clavatus*, *Aspergillus niger*, *Aspergillus nidulans*, *Talaromyces stipitatus* and *Penicillium chrysogenum* showed a KGKAKEXAGEA consensus sequence (Table S1). PREDOTAR, PSORT II, MITOPROT II and TARGETP predict that the protein is targeted to the mitochondria with 79%, 74%, 78% and 91% probability respectively. The open reading frames of the predicted Hsp12 genes, *hsp12A* (NFIA_007970; NCBI-geneID: 4591922) and *hsp12B* (NFIA_058420; NCBI-geneID: 4584904) encode highly hydrophilic proteins of 90 amino acid residues lacking localization signals (Fig. 1) and a glycine content of 8.9% and 14.4% respectively.

LeamA is strongly hydrophilic with a grand average of hydropathicity (GRAVY; Kyte and Doolittle, 1982) value of −0.8, whereas Hsp12A and Hsp12B are even more hydrophilic with values of −1.4 and −1.2 respectively. Like the GRAVY index, the Kyte and Doolittle hydropathy plot revealed that the LeamA protein is strongly hydrophilic, except for the N-terminal pre-sequence that showed hydrophobic properties (Fig. 1B).

FOLDINDEX (Prilusky *et al*., 2005) predicts that the LeamA protein is intrinsically unfolded except for its N-terminal sequence (Fig. S1A). Similarly, Hsp12A and Hsp12B are predicted to be intrinsically unfolded. In contrast, the secondary structure computed with SOPMA, GOR4 and SIMPA96 predict α-helical structure for LeamA (59–75%), Hsp12A (38–49%) and Hsp12B (24–49%) respectively. HELIQUEST predicts that the 11-mer repeat of LeamA forms an amphipathic α-helix similar to that of the helix motif of the mitochondrial LEAM protein from pea (*Pisum sativum*) (Tolleter *et al*., 2007) (Fig. S1B). The α-helices of Hsp12A and Hsp12B also are predicted to have amphipathic properties (Fig. S1B).

Heterologous expression of LeamA, Hsp12A and Hsp12B

LeamA, Hsp12A and Hsp12B were expressed as His-tagged proteins in *Escherichia coli* BL21. Protein bands of approximately 18, 14 and 14 kDa were observed in the Ni-NTA purified protein fractions of BL21–LeamA, BL21–Hsp12A and BL21–Hsp12B respectively (Fig. 2A). These bands were excised from gel and their identity was confirmed by mass spectrometry. Stress tolerance of the strains BL21–LeamA, BL21–Hsp12A, BL21–Hsp12B and the control strain BL21–pET28a against 500 mM NaCl, 500 mM KCl, 130 mM MgCl2 and 1300 mM sorbitol was determined. No difference in growth was observed when the strains were incubated in Luria Broth. The optical density of all cultures was reduced upon exposure to salinity or osmotic stress. However, the OD_{600nm} was significantly higher in cells expressing LeamA, Hsp12A or Hsp12B (Fig. 2B). The OD_{600nm} values of Bl21–Leam were 22.9-, 14.0-, 2.7- and 1.5-fold higher than that of the control strain BL21–pET28a after 20 h of growth in the presence of NaCl, KCl, MgCl2 and sorbitol respectively. These values were 22.0-, 14.3-, 2.7- and 1.6-fold higher in the case of BL21–Hsp12A and 21.4-, 16.7-, 2.8- and 1.6-fold higher in the case of BL21–Hsp12B (Fig. 2B). These results show that heterologous expression of LeamA, Hsp12A and Hsp12B improve salt and osmotic tolerance of *E. coli*.

Protection of lactate dehydrogenase (LDH) enzyme activity

LDH is sensitive to stress conditions such as heat and freeze–thaw cycles (Dong *et al*., 1995). Activity of LDH was monitored in the presence or absence of partially purified LeamA, Hsp12A and Hsp12B (see Fig. 2A) to assess whether these hydrophilins could protect the enzyme. The conversion of pyruvate was measured by a decrease in absorbance at 340 nm due to the oxidation of nicotinamide adenine dinucleotide (di)hydride (NADH). Drying (45 min under vacuum) of LDH alone decreased its activity by 3%, but drying followed by heating for 18 h at 60°C resulted in a 91% decrease in activity. LDH activity after drying and heating was 36%, 35% and 50% of the original activity at a mass ratio of 10:1 (hydrophilin : LDH) and 62%, 73% and 84% at a mass ratio of 40:1 for

A

LeamA

MSSLARFAPLTARVAAVRTTTPAFTAAGSRFISSTPKNEKGPVEATKDTLKKADRVISDNLVK
GIDKGEQAKDKVKQTVGSSTEEAKYKAEGMKEEMKGEASEKAGETKGKASETLGEAKGK
AKEVYGEAKGKAKEMGNM

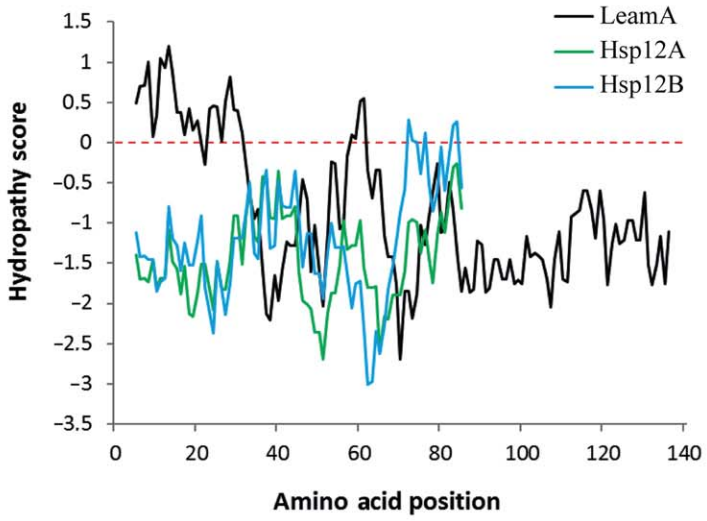
Hsp12A

MSDTGRKDFSTKAQEKMTPDSQKSTMDKMKETVTDATDRLTGSAQGDNQKSYSQQAYDS
VRGETDNQTHGSSTETMGQKAKNAMGLGDH

Hsp12B

MSDAGRKDFSTKAKEEITPDSTKSTQQKIKEGVTDTGDRVARGLQTDGSKSGTQEAFDKTQ
RSHDNHAHGGAGGSIGDKVKNAVGLGNH

B



C

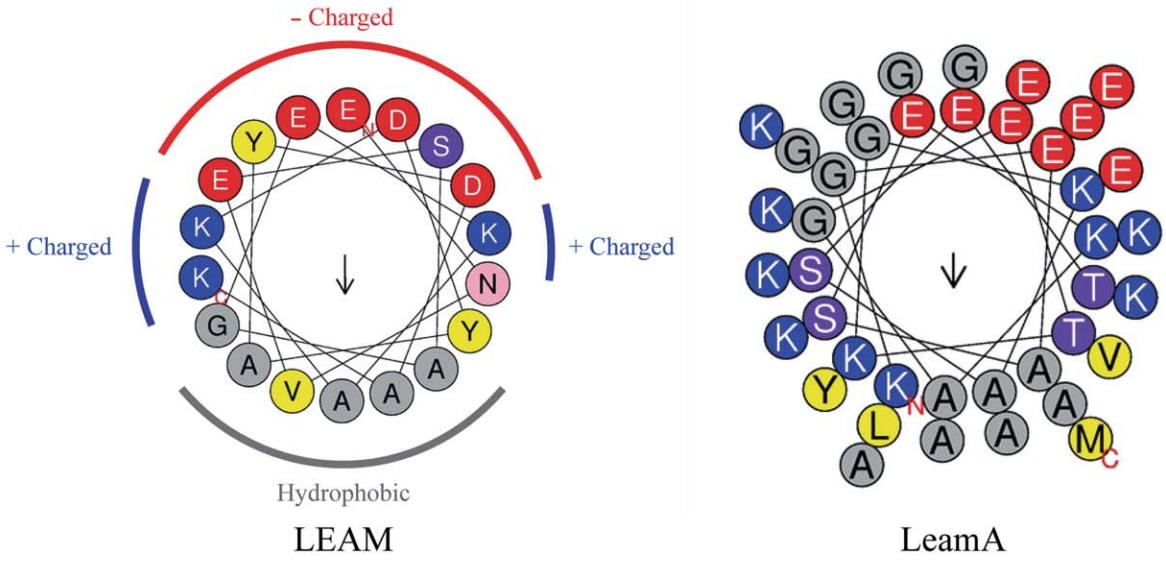


Fig. 1. *In silico* analysis of LeamA, Hsp12A and Hsp12B.
A. Amino acid sequence of LeamA showing the putative mitochondrial localization signal (red) and the C-terminal 11-mer repeats (underlined) and the amino acid sequence of Hsp12A and Hsp12B showing the regions predicted to form α-helices (underlined) of which the largest (red) are represented as a helical wheel projection (see Fig. S1).
B. Hydropathy plot with the amino acids plotted on the x-axis beginning at the N-terminus and using a nine-residue window length. Regions with a hydropathy score below zero are regarded as hydrophilic.
C. Helical wheel projection of the 11-mer repeat of LeamA and the LEAM 101–119 region of *Pisum sativum* (Tolleter *et al.*, 2007) showing the structural analogy between their amphipathic helices.

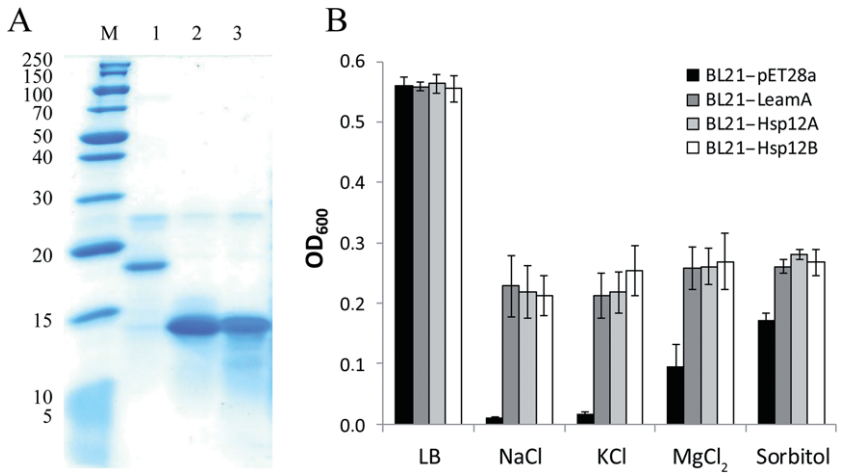


Fig. 2. Heterologous expression of LeamA, Hsp12A and Hsp12B in *Escherichia coli* BL21.
A. The hydrophilins were extracted from BL21–LeamA, BL21–Hsp12A and BL21–Hsp12B cells, partially purified with a Ni-NTA spin column, and separated by SDS-PAGE. LeamA (lane 1), Hsp12A (lane 2) and Hsp12B (lane 3).
B. Growth of BL21–LeamA, BL21–Hsp12A and BL21–Hsp12B in Luria–Bertani or under high salinity (500 mM NaCl, 500 mM KCl or 130 mM $MgCl_2$) or osmolarity (1.3 M sorbitol). OD_{600} as measured after 20 h of growth. Average and standard deviations are based on four independent experiments.

LeamA, Hsp12A, Hsp12B respectively (Fig. 3A). Addition of lysozyme, which has a size similar to the hydrophilins of *N. fischeri*, resulted in 22% and 50% LDH activity after dry heat at a mass ratio of 10:1 and 40:1 respectively. Bovine serum albumin (BSA), a protein known to bind to many proteins, did not protect LDH under these conditions (Fig. 3A).

LDH maintained 42% and 11% of its enzymatic activity after two and six freeze–thaw cycles respectively (Fig. 3B). These values were 91% and 45% when LeamA was present at a 20:1 ratio. Hsp12A, Hsp12B and the cryoprotectant BSA were able to prevent LDH inactivation nearly completely (3%, 1% and 0% loss of activity, respectively, after six freeze–thaw cycles). In contrast, presence of lysozyme resulted in 63% and 17% activity after two or six freeze–thaw cycles respectively (Fig. 3B). These results show that LeamA, Hsp12A and Hsp12B protect LDH against different stresses, whereas lysozyme and BSA only protect against dry heat and freezing–thawing respectively.

Deletion of leamA

We were unable to delete the *hsp12A* and *hsp12B* genes of *N. fischeri*. However, the coding sequence of LeamA of *N. fischeri* was successfully replaced with the hygromycin B resistance cassette using the pAN7-1-Δ*leamA* deletion vector. The absence of *leamA* and the correct integration of pAN7-1-Δ*leamA* were confirmed by polymerase chain reaction (data not shown). Radial extension rates were not affected in the Δ*leamA* strain when compared with the wild type at a growth temperature of 24–50°C (Fig. S2). The optimal and the maximal growth temperatures were 36°C and 45°C, respectively, for both wild type and mutant.

The viability of wild type and Δ*leamA* ascospores was determined after desiccation and heat stress. Ascospores of 40-day-old cultures of the wild-type strain showed similar germination levels after 1 h drying under vacuum when compared with non-dried ascospores (92% and 93% respectively). The survival rate was 78% when the dried ascospores had been heated for 1 h at 60°C. This decreased to 74% and 64% when the spores had been incubated at 70°C and 80°C respectively (Fig. 4). Drying of Δ*leamA* ascospores of 40-day-old cultures resulted in a viability of 58%, whereas viability of the untreated control was 82%. Drying followed by heat shock (60, 70 and 80°C) resulted in survival of Δ*leamA* ascospores of 36%, 28% and 16%, respectively (Fig. 4). Taken together, these data show that LeamA promotes survival during exposure to drought and heat.

Discussion

The role of the hydrophilins LeamA, Hsp12A and Hsp12B of *N. fischeri* was studied in relation to stress resistance. This is the first functional characterization of such proteins derived from a filamentous fungus. Glycine content, hydrophilicity and the propensity to fold into amphipathic α-helical structure classify LeamA, Hsp12A and Hsp12B of *N. fischeri* as hydrophilins (Dure, 1993a,b; Garay-Arroyo *et al.*, 2000; Battaglia *et al.*, 2008). The consensus sequence of the C-terminal repeat of LeamA (KGKAKEXAGEA) is predicted to form an amphipathic α-helix. Similarly, Hsp12A and Hsp12B are predicted to form amphipathic α-helical structures. It has to be expected that like other hydrophilins, these proteins are intrinsically unfolded in solution (see also Uversky *et al.*, 2000), but obtain secondary structure upon drying or interaction with proteins or lipids.

An N-terminal stretch of 39 amino acids predicts that LeamA resides in the mitochondria. Mitochondrial-localized LEA-like proteins have been identified earlier in the brine shrimp *Artemia franciscana* (Menze *et al.*, 2009) and in pea (*P. sativum*) (Grelet *et al.*, 2005; Tolleter *et al.*,

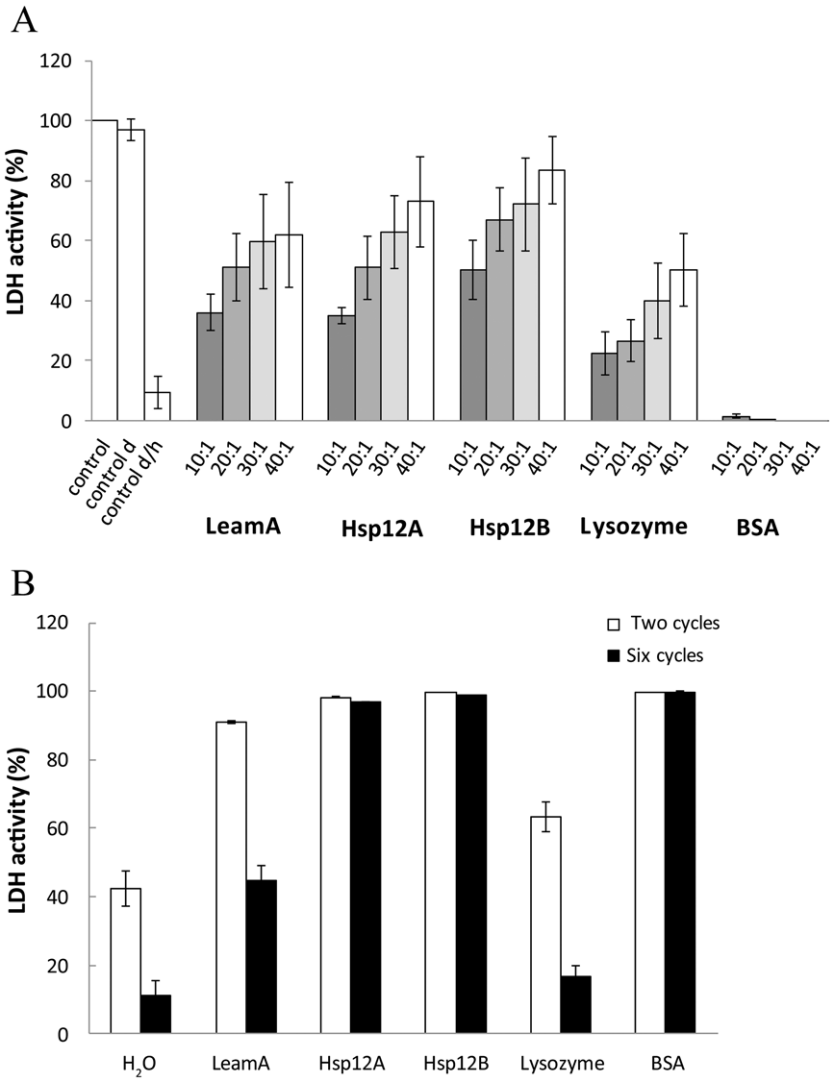


Fig. 3. LDH protection by LeamA, Hsp12A and Hsp12B against heat and cycles of freezing in liquid nitrogen and thawing at ambient temperature.
A. Relative residual LDH activity was measured at OD_{340} after drying and exposure at 60°C for 18 h in the absence or presence of LeamA, Hsp12A and Hsp12B in various ratios (protein protectant : LDH). The control was maintained at 7°C, dried (d), or dried and heated (d/h).
B. Relative residual LDH activity after two and six freeze–thaw cycles in the absence or presence of LeamA, Hsp12A and Hsp12B (protein protectant : LDH ratio 20:1). Lysozyme and BSA served as controls.

2007). Hsp12A and Hsp12B lack localization signals and will be released into the cytosol upon synthesis. An Hsp12 of *S. cerevisiae* was found in the cytoplasm, but at later growth stages interacted with the plasma membrane and endosomes (Welker *et al*., 2010) and thereby might help in stabilizing these structures leading to lifespan extension (Herbert *et al*., 2012). This protein however also has been shown to reside in the cell wall (Motshwene *et al*., 2004), enabling the protein to protect both sides of the plasma membrane. Together, LeamA and the Hsp12 proteins would protect the mitochondrial and plasma membranes of *N. fischeri*, respectively to alleviate stress occurring upon drying (Tolleter *et al*., 2007; Welker *et al*., 2010; Herbert *et al*., 2012).

The hydrophobic and hydrophilic sides of the amphiphilic α-helices are assumed to interact with the membrane core and the negatively charged heads respectively (Tolleter *et al*., 2007; 2010). By doing so, they would protect membranes. PsLEAm of pea enhances stability of liposomes against desiccation, rehydration and freezing. Its protective capacity depends on phospholipid composition. The mitochondrial lipid cardiolipin protects membranes in a similar way (Tolleter *et al*., 2010).

LeamA, Hsp12A and Hsp12B of *N. fischeri* also protected the protein LDH during desiccation, heat and freeze–thaw cycles. Similarly, PsLEAm of pea protects the mitochondrial matrix enzymes fumarase and rhodanese against desiccation (Grelet *et al*., 2005).

Deletion of *leamA* increased the sensitivity of *N. fischeri* ascospores to a combination of drought and heat. Presence of this protein in the ascospores should be confirmed in future studies. Transcripts of the homologue of *leamA* of *A. niger* (Accession number: A2QDJ8_ASPNC; Table S2) are highly present in dormant conidia (van Leeuwen *et al*., 2013a,b), but its mRNA levels rapidly decreases at initiation of germination. Transcripts of the

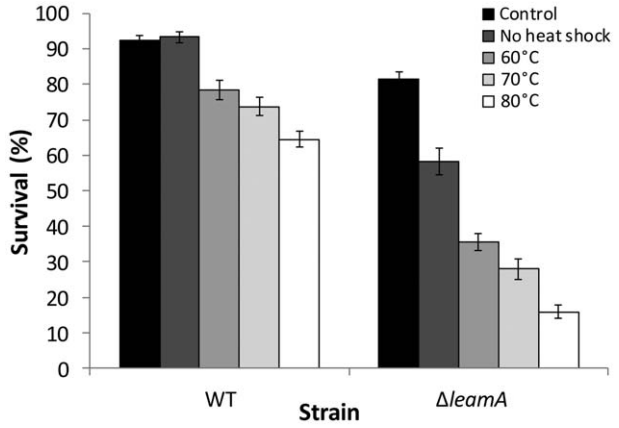


Fig. 4. Heat and drought tolerance of Δ*leamA*. Ascospores of wild type (WT) and Δ*leamA* were dried and either subjected or not to a 1 h dry heat shock at 60, 70 or 80°C followed by heat activation for 2 min at 85°C in an aqueous solution. Average and standard deviations are based on biological triplicates. 'Control' are non-dried ascospores not subjected to a dry heat shock. 'No heat shock' are dried ascospores not subjected to a dry heat shock.

hydrophilin gene *con-6* also accumulate in conidia, ascospores and microconidia of *Neurospora crassa* (Springer and Yanofsky, 1992; White and Yanofsky, 1993). The level of the encoded protein rapidly decreases within 2 h of germination and is undetectable after 16 h (White and Yanofsky, 1993). That hydrophilins can help protect fungal survival structures is suggested by the accumulation of transcripts of homologues of a LEA-like protein, a small HSP and two dehydrins in dormant conidia of the fungus *A. niger* (van Leeuwen *et al*., 2013a). Also, conidia stressed with an antifungal compound, natamycin, showed marked up-regulation of these transcripts (van Leeuwen *et al*., 2013b).

We have not been able to inactivate *hsp12A* and *hsp12B* in *N. fischeri* and this may indicate that these genes are essential. It would be of high interest to evaluate if compatible solutes such as trehalose and trehalose-based oligosaccharides that are recently isolated from ascospores of *N. fischeri* (Wyatt *et al*., 2015a,b) function synergistically with the cytoplasmic Hsp12A and Hsp12B proteins.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. *In silico* analysis of LeamA, Hsp12A and Hsp12B.
A. Degree of protein disorder according to the web-based FOLDINDEX application showing unfolded (FoldIndex < 0) and folded (FoldIndex > 0) regions.
B. Helical wheel projection of the largest α-helices forming regions of Hsp12A and Hsp12B (designated in red in Fig. 1).

Fig. S2. Heat and drought tolerance of Δ*leamA*. Colonies of wild type (WT) and Δ*leamA* were grown from mycelial plugs on oatmeal agar at a temperature varying from 24 to 50°C.
Table S1. Alignment of the 11-mer repeat sequence of the LEA-like proteins of ascomycetes related to *N. fischeri* and their consensus sequence.
Table S2. Primers used in this study.
Appendix S1. Experimental procedures.