Journal of Molecular Evolution (2019) 87:289–297
https://doi.org/10.1007/s00239-019-09900-9

ORIGINAL ARTICLE

Stress Adapted Mollusca and Nematoda Exhibit Convergently Expanded Hsp70 and AIG1 Gene Families

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Received: 29 April 2019 / Accepted: 1 July 2019 / Published online: 5 September 2019

Abstract

We recently sequenced the genome of the first subterrestrial metazoan, the nematode *Halicephalobus mephisto*. A central finding was a dramatic expansion of genes encoding *avrRpt2* induced gene (AIG1), and 70 kDa heat shock (Hsp70) domains. While the role of Hsp70 in thermotolerance is well established, the contribution of AIG1 is much more poorly characterized, though in plants some members of this family are heat-induced. Hypothesizing that this dual domain expansion may constitute a general biosignature of thermal stress adaptation, here we examine a number of genomes, finding that expansion of both AIG1 and Hsp70 is common in bivalves. Phylogenetic analysis reveals that the bivalve-specific Hsp70 protein expansion groups with *H. mephisto* sequences. Our identification of the same gene expansions in bivalves and a nematode implies that this biosignature may be a general stress adaptation strategy for protostomes, particularly those organisms that cannot escape their stressful environments. We hypothesize that the two families play largely complementary mechanistic roles, with Hsp70 directly refolding heat-denatured proteins while AIG1 promotes cellular and organismal survival by inhibiting apoptosis.

Keywords Hsp70 · AIG1 · Bivalve · Mollusca · ER stress · Thermotolerance

Introduction

The evolutionary adaptation to thermal stress is a phenomenon of increasing importance as the planet warms. Genomic adaptation to long-term warming must be distinguished from the more well-characterized heat shock response to brief extreme temperature spikes that would be lethal if sustained over a longer period of time. In contrast, long-term heat induces both physiological and genomic changes (Porcelli et al. 2015). The adaptation to long-term heat stress is critically dependent on escapability, with genomic adaptation occurring when organisms cannot flee and must instead adapt or die.

Despite being distinct phenomena, heat shock and constitutive heat adaptation have been found to share some mechanistic similarities both at the organismal and molecular levels. For example, 70 kDa Heat Shock Proteins (Hsp70) is a family of proteins shared between the two phenomena. These proteins are chaperones involved in the refolding of denatured proteins including those damaged by heat stress (Sung et al. 2018). Hsp70 proteins also respond to a variety of environmental stressors such as hypoxic conditions, oxidative stress, altered pH, exposure to heavy metals, parasites or infections, and especially elevated temperatures (Mizrahi et al. 2011). Under extreme stressors, the survival of the organism depends on Hsp70's ability to fix denatured and misfolded proteins (Murphy 2013). While acute heat shock generally involves dramatically induced expression of a single or few Hsp70 genes, long-term heat adaptation works differently, with the Hsp70 genes being expressed at low levels to minimize long-term cytotoxicity of these proteins (Sorensen et al. 2001, 2003).

Handling Editor: **David Liberles**.

Electronic supplementary material The online version of this article (doi:https://doi.org/10.1007/s00239-019-09900-9) contains supplementary material, which is available to authorized users.

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The Biosignature of Genomic Adaptation to Stress

We recently published the genome and transcriptome of a nematode, *Halicephalobus mephisto,* that lives 1.3 km underground in the deep terrestrial subsurface and is adapted to chronic heat stress, living in hypoxic 37 °C water in the deep terrestrial subsurface (Borgonie et al. 2011; Weinstein et al. 2019). By analyzing the genome of *H. mephisto* we identified an apparent signature of adaptation: expanded copy number of Hsp70 and the *avrRpt2*-Induced Gene (AIG1) family. While Hsp70s are extremely well characterized in relation to heat, AIG1 is less well characterized, having been initially identified as a pathogen response gene in *Arabidopsis thaliana* (Reuber and Ausubel 1996) and found to be involved in responding to both biotic and abiotic stresses including heat (Liu et al. 2008). In mammals AIG1 are also called Immune Associated Nucleotide (IANs) genes or GTPases of Immunity Associated Proteins (GIMAPs) and have been shown to play critical roles in the immune education of T lymphocytes (Poirier et al. 1999; Krucken et al. 2004; Nitta et al. 2006). In both Pacific and pearl oyster genomes, the Hsp70 gene family was found to be expanded (Zhang et al. 2012; Takeuchi et al. 2016) and the Sydney Rock Oyster (*S. glomerata*) encodes an expansion of both Hsp70 and AIG1 (Powell et al. 2018).

Given that these three oysters display pronounced thermotolerance (Zhang et al. 2012; Liu et al. 2014; Ertl et al. 2016) (Table 1), we hypothesized that expanded Hsp70 and AIG1 gene families together produce effective protection against thermal stress and that we would be able to identify this signature in other species. Therefore, we performed genomic scans in a range of organisms including other molluscs, other animals, plants, and bacterial outgroups. To identify Hsp70 and AIG1 genes, we performed a Hidden Markov Model search (Eddy 2011) of published proteomes. Several patterns are immediately apparent in the results (Table 1). First, it appears our methodology captures most or all known Hsp70 genes, as in humans 17 have been reported; we identified 19 (Table 1). While *C. gigas* has been reported to have 88 Hsp70 genes (Zhang et al. 2012), we found 101 (Table 1), suggesting that the Pacific Oyster genome annotation has been updated since 2012 or that our detection threshold may be more liberal. A second striking feature is the high variability in gene content for the Hsp70 and AIG1 families across taxonomic groups, possibly reflecting variable selective pressures for adaptation to stress.

Consistent with this hypothesis, a strong tendency for AIG1 and Hsp70 genes to co-expand was observed in a number of lineages, particularly within bivalves, which as a whole are known to be stress-resilient (Table 1). In six of seven examined bivalves, Hsp70 and AIG1 were present at copy numbers comparable to *H. mephisto* (Table 1). Both *Pinctada fucata* and *Crassostrea gigas* have been reported to have expanded Hsp70 (Zhang et al. 2012; Takeuchi et al. 2016), but we show they encode expanded AIG1 gene repertoires also (Table 1). The Pacific Oyster inhabits intertidal zones where it experiences considerable and variable heat and desiccation stress (Zhang et al. 2012), but all bivalves undergo significant environmental challenges including salinity, pathogenic and anaerobic stress compounded by a sessile lifestyle, minimizing ability to escape (Takeuchi et al. 2016; Powell et al. 2018).

In contrast to bivalves, we find that gastropods have consistently lower Hsp70 numbers (10–12 copies) but two species exhibit extremely amplified AIG1 gene families: the ram's horn snail *Biomphalaria glabrata* and the golden apple snail *Pomacea canaliculata* (Table 1). *B. glabrata* apparently grows well for weeks at 33 °C (Nelson et al. 2016). This organism is an obligate intermediate host of the schistosomiasis parasite *Schistosoma mansoni*, suggesting that its expanded AIG1 genes may respond to this biotic stress. Interestingly, at least one Hsp70 gene has been found to respond to infection with *S. mansoni* (Ittiprasert et al. 2010); nothing is known regarding involvement of AIG1 in this process. The golden apple snail *Pomacea canaliculata* has both thermal (Seuffert and Martín 2013) and cold tolerance (Matsukura et al. 2009) but nothing is known about the response of its AIG1 genes to either abiotic stress, though its Hsp70 genes were shown to be to over 20-fold induced under heat shock (Liu et al. 2018).

The basal lophotrochozoan brachiopod *Lingula anatina* (Luo et al. 2015) has 16 and 14 genes for Hsp70 and AIG1, respectively, which suggests the Hsp70 and AIG1 gene family expansions occurred in the bivalve lineage specifically, while the gastropod lineage underwent a mild reduction of Hsp70 gene number to 10–12 copies, consistent with a previous report (Takeuchi et al. 2016). The only cephalopod we examined, the octopus *O. bimaculoides,* had only 3 AIG1 and 11 Hsp70 genes detected (Table 1).

Phylogenetic Analysis of Hsp70

For phylogenetic analysis, we chose Hsp70 since these genes are easy to identify, highly conserved, and well studied (Feder and Hofmann 1999; Radons 2016). This rich background knowledge makes phylogenetic analysis informative, with many isoforms well characterized in terms of compartmentalization and overall function (Feder and Hofmann 1999; Radons 2016) and raising the question of whether the bivalve-expanded sequences are from well-studied Hsp70 subfamilies. In contrast, AIG1 genes are much more recently

Table 1 A survey of Hsp70 and AIG1 gene copy number in 37 genomes

Organism	Phylum	Higher classification	HSP70	AIG1	Thermotolerant?	Max growth temp	Other stress resistant?	Reference for growth temp	**Reference for other stress**
P. lepturus	Chordata	Vertebrate	12	5					
H. sapiens	Chordata	Vertebrate	19	13					
D. gigantea	Cnidaria	Coral	7	21					
A. planci	Echinodermata	Crown of thorns starfish	13	9					
S. purpuratus	Echinodermata	Echinoidea	13	1	Yes	32		Hammond and Hofmann (2010)	
H. robusta	Annelida	Leech	8	4					
C. teleta	Annelida	Polychaeta	17	0					
L. anatina	Brachiopoda	Basal lophotrochozoan	16	14					
C. giggas	Mollusca	Bivalve	101	47	Yes	50	Hypoxia	Zhang et al. (2012)	David et al. (2005) and Sussaralu et al. (2010)
P. fucata	Mollusca	Bivalve	68	43	Yes	32		Liu et al. (2014)	
P. yessoensis	Mollusca	Bivalve	61	33	No	25		Chen et al. (2007)	
C. virginica	Mollusca	Bivalve	113	81	Yes	30–35	Hypoxia	Heilmayer et al (2008) and Stanley et al. (1986)	Stickle et al. (1989)
M. galloprovincialis	Mollusca	Bivalve	20	6	Intermediate	30		Hoffmann et al. (1996)	
S. glomerata	Mollusca	Bivalve	88	62	Intermediate	30		Ertl et al. (2016)	
B. platifrons	Mollusca	Bivalve	103	44			Low temp., hypoxia, methane		Sun et al. (2017) and Wong et al. (2015)
O. bimaculoides	Mollusca	Cephalopod	11	3					
E. chlorotica	Mollusca	Gastropod	12	21					
L. gigantea	Mollusca	Gastropod	10	14					
P. canaliculata	Mollusca	Gastropod	11	94	Yes	35	Low temp.	Seuffert et al. (2013)	Matsukura et al. (2009)
A. californica	Mollusca	Gastropod	11	31					
B. glabrata	Mollusca	Gastropod	10	106	Yes	33		Nelson et al. (2016)	
C. elegans	Nematoda	Roundworm	12	1	No	25		Zhang et al. (2015)	
D. coronatus	Nematoda	Roundworm	260	0					
D. pachys	Nematoda	Roundworm	102	2	Intermediate	30		Gibbs et al. (2005) and Lemzina and Gagarin (1994)	
H. mephisto	Nematoda	Roundworm	86	60	Yes	40		Weinstein et al. (2019)	
P. redivivus	Nematoda	Roundworm	24	11					
B. anynana	Arthropod	Insect	12	3					
T. castaneum	Arthropod	Insect	12	4					
A. thaliana	Plantae	Brassicaceae	17	25					
C. rubella	Plantae	Brassicaceae	13	23					
A. lyrata	Plantae	Brassicaceae	12	21					
T. hassleriana	Plantae	Cleomaceae	20	17					
P. patens	Plantae	Earthmoss	26	19					
V. splendidus	Bacteria	Proteobacteria	5	5					
V. tubiashii	Bacteria	Proteobacteria	6	3					
A. fischeri	Bacteria	Proteobacteria	5	2					
S. pneumoninae	Bacteria	Fermicutes	1	3					

Hsp70 and AIG1 gene copy numbers are displayed in a heat map ranging from green (lower) to red (higher) abundances. Higher classification categories are indicated as red for vertebrates, purple for cnidaria, dark green for echinoidea, tan for annelids, yellow for brachiopods, light blue for bivalves, orange for cephalopods, neon green for gastropods, dark pink for roundworms, light purple for insects, light green for plants, and dark blue for bacteria. The same color scheme was used in the phylogenetic tree of Fig. 1

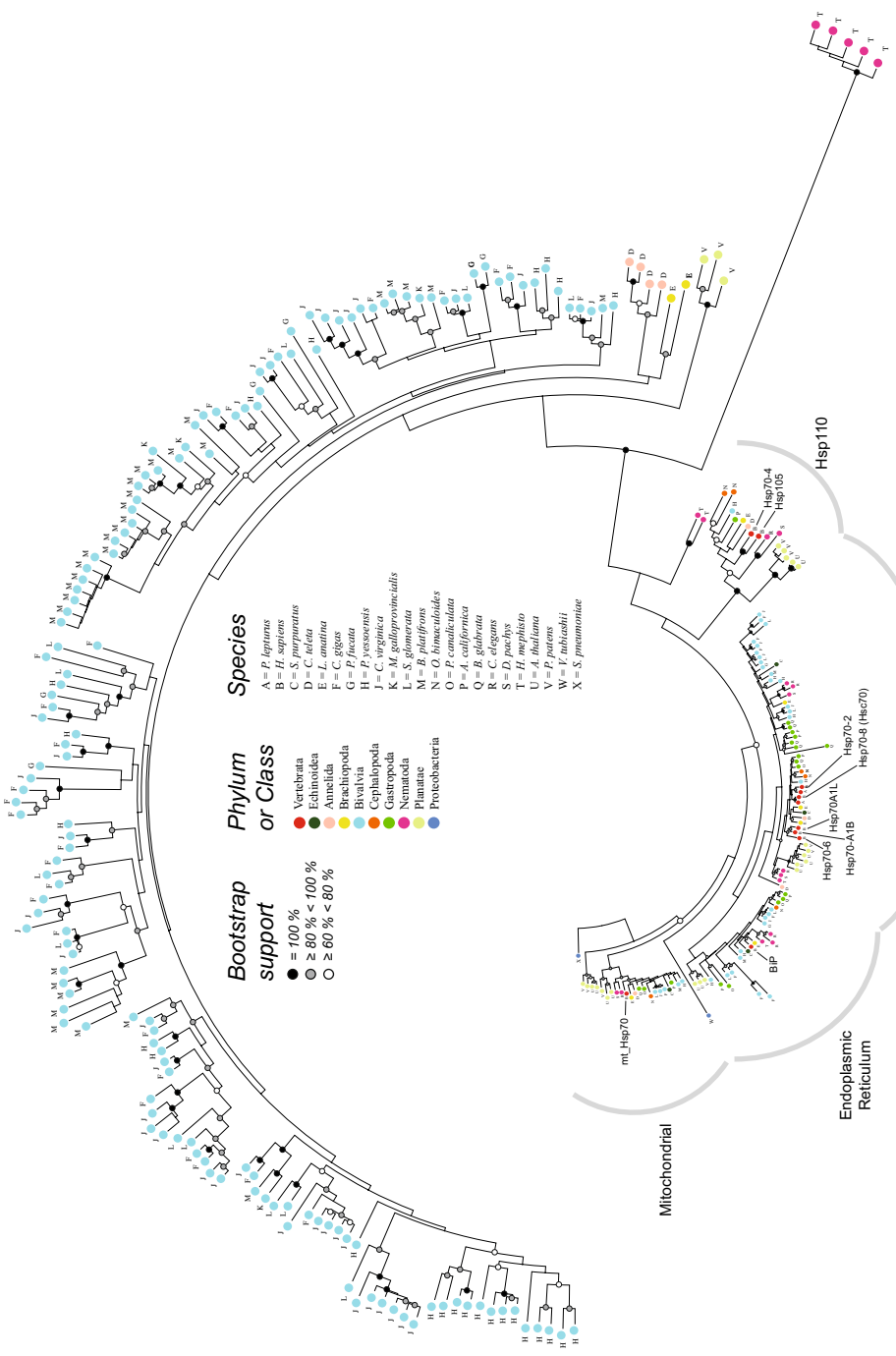

Fig. 1 A maximum-likelihood of 279 Hsp70 sequences (666 sites) across 23 species. Colored dots indicate phylum or class while single-letter codes indicate species, as shown in the figure legend. Dots placed on nodes indicate bootstrap support with solid black fill indicating 100%, gray fill indicating ≥ 80% < 100%, while open circles on nodes indicate ≥ 60% < 80%. Known clades of Hsp70 genes are annotated at the bottom of the figure. The alignment is provided as supplemental data with this manuscript

discovered and phylogenetically nest within a very large but poorly characterized family of GTPase proteins making phylogenetic analysis both more difficult and less informative (Weinstein et al. 2019).

Therefore, to assess whether the expanded Hsp70 genes of bivalves reflect expansion of known Hsp70 families (Radons 2016) we constructed a maximum-likelihood tree using 279 Hsp70 genes from 23 species including all 7 bivalves from Table 1 (Fig. 1). To enable maximal inclusion of bivalve sequences we only included 9 *H. mephisto* sequences. The bivalve Hsp70 expansion does not group with known Hsp70 genes, but are instead most closely related to sequences also found in the annelid *C. teleta* (Simakov et al. 2013), the basal lophotrochozoan brachiopod *L. anatina* (Luo et al. 2015) and the moss *P. patens* (Rensing et al. 2008). This suggests an undescribed subfamily of Hsp70 genes exists within lophotrochozoan and plant genomes and has radically expanded within the bivalve lineage, consistent with a previous report (Takeuchi et al. 2016) (Fig. 1). Surprisingly, this bivalve-expanded Hsp70 subfamily groups with the *H. mephisto* sequences with 100% bootstrap support (Fig. 1). Whether this reflects expansion of an ancestral sequence within protostomes and mosses, or convergent evolution, is currently unclear. However, many bivalves and *H. mephisto* are heat-tolerant species and the expansion of Hsp70 genes in moss is hypothesized as an adaptation to heat and desiccation stress encountered during the transition to life on land (Rensing et al. 2008). Our data may represent a particularly striking case of molecular convergent evolution in which the same widely conserved Hsp70 family member is independently expanded in multiple lineages to mitigate environmental heat stress. Consistent with natural selection favoring the expansion of these genes, our phylogenetic tree reveals recent species-specific Hsp70 expansions for *P. yessoensis, C. virginica,* and *B. platifrons,* along with older mixed-species clusters dating to ancestral bivalve lineages (Fig. 1).

Functional Implications of the Biosignature

The precise functional role of the genomic signature—expanded Hsp70 and AIG1—in bivalves remains unclear. While heat induces expression of Hsp70 genes in Pacific oyster (Zhang et al. 2012), its AIG1 gene expansion has not previously been documented; it has been shown that hypoxia also induces Hsp70 gene expression in this organism (David et al. 2005). In pearl oysters, Hsp70 was shown to be upregulated under heat stress (32 °C) (Liu et al. 2014) but these genes are also involved in pearl biomineralization (Du et al. 2017; Mariom et al. 2019). Bivalves must also contend with infectious threats due to their sessile lifestyle (Ding et al. 2015; Takeuchi et al. 2016), raising the possibility that Hsp70 or AIG1 may interface with the immune system of these organisms. Consistent with this, the eastern oyster *C. virginica* was shown to upregulate Hsp70 in response to bacterial pathogens (McDowell et al. 2014). However, it can tolerate a wide range of temperatures in its varied habitat along the Atlantic seacoast, up to 30–35 °C (Stanley 1986; Heilmayer et al. 2008); in addition it is strongly hypoxia-resistant (Stickle et al. 1989). It would be intriguing to examine heat- and hypoxia-mediated changes in gene expression in this organism to see whether AIG1 or Hsp70 play a role in responding to these abiotic stresses.

The role of Hsp70 and AIG1 in stress response of *H. mephisto* appears to be complex. We found that Hsp70 is upregulated under heat stress in the laboratory, but AIG1 genes were not induced under thermal conditions, leading us to hypothesize these genes are involved in hypoxia or other environmental stress (Weinstein et al. 2019). Therefore, it is possible that the shared pattern of gene expansions seen in *H. mephisto* and bivalves reflect multiple intersecting adaptive pressures, not just heat stress.

Elevated heat causes an accumulation of non-secreted, misfolded proteins within the ER, creating ER stress. If not remedied, the cell will go through apoptosis (Oakes and Papa 2015). The unfolded protein response (UPR) is a conserved adaptive mechanism to ER stress which aims to restore protein homeostasis and promote cell survival (Sano and Reed 2013; Oakes and Papa 2015). Therefore, AIG1 may promote cell survival until the UPR relieves cellular ER stress. While AIG1 genes were not induced under thermal stress in *H. mephisto,* several ER stress and the UPR genes were upregulated, such as Bax Inhibitor-1 (BI-1) (Weinstein et al. 2019). In addition to its role as an inhibitor of the pro-apoptotic gene Bax (Xu and Reed 1998; Cai et al. 2018), BI-1 also forms a complex with the ER stress gene IRE1α, nullifying its endonuclease activity, suppressing the apoptotic UPR pathway and promoting cell survival (Sano and Reed 2013). Another gene we uncovered involved with UPR response is arginine-rich, mutated in early-stage tumors (ARMET), a gene whose precise function has not yet been defined but that responds to ER stress (Mizobuchi et al. 2007; Murphy 2013). Within the ER ARMET interacts directly with ER Hsp70 protein BiP/GRP78 (Glembotski et al. 2012). These data suggest that a combination of (1) Hsp70 and (2) inhibiting ER stress-located apoptotic pathways are important in heat resilience. AIG1 can perform the ER stress-induced apoptosis blocking: in rats an ER-located AIG1 gene, GIMAP5, was shown to inhibit ER stress-induced apoptosis (Pino et al. 2009).

We note that heat-mediated induction of AIG1 expression may not be required in the case of *H. mephisto,* which has 60 copies, effectively creating a huge constitutive increase of expression relative to other nematodes, which range from 0 to 4 copies (Table 1). While heat did not induce AIG1 expression, neither did we see a reduction in expression at

low temperatures (Weinstein et al. 2019) so AIG1 simply remains constitutively expressed from its multiple copies regardless of culture temperature. In marked contrast, Hsp70 gene expression comes at an overall cost to organismal development, fertility, and survival, apparently because the ATP-requiring chaperone diverts energy that could otherwise go to reproduction or development (Feder and Hofmann 1999). Therefore, at lower temperatures when Hsp70 is not needed, its expression is strongly downregulated (Weinstein et al. 2019); we speculate that AIG1 may not engender the same fitness trade-off so natural selection has not produced a thermal regulatory mechanism (yet).

An informative natural experiment on the relationship between Hsp70 and AIG1 expansions exists in *Diploscapter pachys* and *D. coronatus*, close relatives *C. elegans*, which carry a massive expansion of Hsp70 genes, but not the AIG1 family (Weinstein et al. 2019) (Table 1). In contrast to *H. mephisto*, the thermotolerance of *Diploscapter* species is moderate: they have been isolated from thermal waters (Lemzina and Gagarin 1994), can grow up to 30 °C in warm soils (Gibbs et al. 2005), and the maximum growth temperature for *D. pachys* in our laboratory was also 30 °C (our unpublished observations). These temperatures are higher than the *C. elegans* maximum growth temperature of 25 °C; however, *H. mephisto* survive up to 10° higher than *D. pachys* (Weinstein et al. 2019). These data suggest that AIG1 gene duplications may play a critical synergistic role in heat stress, a function that may apply also to bivalves. Supporting this, the Yesso scallop *Patinopecten yessoensis* has fewer AIG1 genes (33) than most bivalves (median AIG1 number = 44) (Table 1) and it was the most sensitive to heat, with a maximum growth temperature of 25 °C (Chen et al. 2007). A curious anomaly in our data is the mediterranean mussel *Mytilus galloprovincialis* which only apparently contains 20 Hsp70 and 6 AIG1 genes, far less than its bivalve relatives yet displays moderate thermotolerance, growing at 30 °C (Hofmann and Somero 1996) (Table 1). To investigate this further, we performed a preliminary blast analysis to search for potentially un-annotated Hsp70 and AIG1 genes in the genome, and found a conservative estimate of 186–263 Hsp70 and 42–52 AIG1 members depending on bivalve species used as query (see "Methods" for details). We therefore conclude that the mediterranean mussel in fact encodes an ensemble of Hsp70 and AIG1 similar to its bivalve relatives, though a more thorough re-annotation effort will be needed to gain more accurate gene counts than we can provide here.

Among the species harboring the Hsp70 and AIG1 gene expansion was *Bathymodiolus platifrons,* a mussel common at 'cold seeps': areas in the cold deep ocean where methane and toxic hydrocarbons leach from the ocean floor, temperatures are cold, pressure is high, and the organism faces exposure to heavy metals along with the hydrocarbons (Levin et al. 2016). *B. platifrons* harbors endosymbiotic bacteria which metabolize methane and hydrogen sulfide while also providing the mussel with food (Won et al. 2003; Duperron et al. 2005). The sequenced mussel sample was from 1.1 km in depth (Sun et al. 2017), similar to the depth of *H. mephisto* at 1.3 km underground (Borgonie et al. 2011). The environment may share similarity in terms of oxygen deprivation, exacerbated by the oxygen-consuming activity of chemolithoautotrophic endosymbionts (Jannasch and Mottl 1985), though not in temperature, which varies in opposite directions–the deep ocean is cold while the terrestrial subsurface is hot, suggesting the genomic signature may contribute tolerance to temperature extremes in general.

Our approach has limitations. The gene counts in Table 1 likely provide a conservative underestimate of gene expansion because highly similar (recent) gene copies falling within the 90% clustering criteria are collapsed into one non-redundant copy. Consistent with this we observed that *B. platifrons* was originally reported to have 179 Hsp70 proteins (Sun et al. 2017) and we identified 103 genes; similarly, here we report 88 Hsp70 genes in *H. mephisto* when originally we found 112 genes, some of which were very similar to each other (Weinstein et al. 2019). In Fig. 1, Hsp70-A1A was removed due to its similarity to Hsp70-A1B, from which it differs only at 2 amino acids (Radons 2016). Secondly, we have no data on actual gene expression of these genes outside *H. mephisto*. Some genomic studies lack transcriptome data entirely, or samples from different growth temperatures have not been obtained. In some cases, the RNA-seq data have not been deposited yet in public databases. Therefore, the expansion of Hsp70 and AIG1 gene copy number is strictly a genomic adaptive signature awaiting a thorough analysis of transcriptional regulation in the future.

Conclusions

Our work on a subterrestrial nematode uncovered a biosignature that we show is also widespread among bivalves, suggesting a genomic mode of adaptation that occurs when environmental stress cannot be avoided. We also show that the Hsp70 expansion appears to be widely conserved, or convergently expanded, in bivalves, an annelid, a basal lophotrochozoan, the nematode *H. mephisto*, and a moss. Collectively expanding Hsp70 and AIG1 creates a genomic signature for adaptation to heat in the deep subsurface (*H. mephisto)*, cold toxic seeps in the deep ocean (*B. platifrons*), hot or anaerobic conditions along with pathogens (bivalves) and perhaps even to adapting to life on land (*P. patens*). If this is the case, this pattern may provide a highly effective general strategy for adaptation to environmental stresses, of increasing importance during coming periods of climatic change.

Methods

Protein Identification

Protein sequences were downloaded from RefSeq or other public repository as appropriate (see Accession Numbers subheader). We used the hmmsearch command from HMMER v3.2.1 (Eddy 2011) to identify Hsp70 or AIG1-domain containing proteins at an e-value of 1e−4, saving the results as a domain table (–domtblout). (Note: an e-value of 1e−4 was chosen because the largest e-value detected from scanning the known human Hsp70 genes was 1e−5.)

Protein Clustering

To remove redundant annotations or alternative splicing isoforms we clustered the proteins at 90% identity within each species using UCLUST v 11.0.667 (Edgar 2010). Before clustering we sorted sequences by length using the -sortbylength option, along with -minsequencelength to remove sequences below 400 aa for Hsp70 and 200 aa for AIG1. Clustering was performed using the -cluster_fast option with 90% identity (specified as -id 0.9). The resulting non-redundant protein (centroid) set was used in both the table and phylogenetic tree analysis by maximum likelihood.

Protein Alignment and Tree Building

For the tree, non-redundant Hsp70 protein sequences from 23 species were obtained as described above were aligned with MAFFT v7.017 (Katoh et al. 2002) and manually refined to remove problematic sequences. The original alignment had 1,119 positions aligned across 279 protein sequences, and Gblocks v 0.19b (Castresana 2000) was used to remove any poor-quality regions of the alignment, leaving 666 informative positions (59%) for inferring the tree. (Gblocks parameters were: Minimum number of sequences for a conserved position: 140; Minimum number of sequences for a flank position: 250; Maximum number of contiguous non-conserved positions: 100; Minimum length of a block: 3; Allowed gap positions: all.) The alignment is provided as a supplemental fasta file (Supplemental Fasta 1). To build the tree, RAxML version 8.2.12 (Stamatakis 2014) was used with the PROTCATBLOSUM62 rate matrix for 200 bootstrap replicates.

Evaluation of *M. galloprovincialis*

To evaluate potentially un-documented Hsp70 and AIG1 genes in the *M. galloprovincialis* genome, we used using the Hsp70 and AIG1 centroids from three bivalves species, *P. yessoensis*, *C. gigas*, and *P. fucata* as query in tblastn (Altschul et al. 1990) against the *M. galloprovincialis* genome with an e-value of 1e−50, giving output in tabular format (-outfmt 6). A custom python script was used to parse the unique loci requiring them be at least 10 kb apart, else they were merged. We found that using different bivalve centroids as tblastn query had only minor effects on gene estimates: Hsp70 gene estimates were 186, 187, and 263 while AIG1 was 42, 52, and 51 with *P. yessoensis*, *C. gigas*, and *P. fucata* queries, respectively.

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