The Journal of Experimental Biology 214, 59-68

doi:10.1242/jeb.050328

RESEARCH ARTICLE

Foreign genes and novel hydrophilic protein genes participate in the desiccation response of the bdelloid rotifer *Adineta ricciae*

Chiara Boschetti, Natalia Pouchkina-Stantcheva*, Pia Hoffmann and Alan Tunnacliffe†

Department of Chemical Engineering and Biotechnology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QT, UK

*Present address: 712 Darwin Centre, Department of Zoology, The Natural History Museum, Cromwell Road, London SW7 5BD, UK

†Author for correspondence (at10004@cam.ac.uk)

Accepted 27 September 2010

SUMMARY

Bdelloid rotifers are aquatic micro-invertebrates with the ability to survive extreme desiccation, or anhydrobiosis, at any life stage. To gain insight into the molecular mechanisms used by bdelloids during anhydrobiosis, we constructed a cDNA library enriched for genes that are upregulated in *Adineta ricciae* 24 h after onset of dehydration. Resulting expressed sequence tags (ESTs) were analysed and sequences grouped into categories according to their probable identity. Of 75 unique sequences, approximately half (36) were similar to known genes from other species. These included genes encoding an unusual group 3 late embryogenesis abundant protein, and a number of other stress-related and DNA repair proteins. Open reading frames from a further 39 novel sequences, without counterparts in the database, were screened for the characteristics of intrinsically disordered proteins, i.e. hydrophilicity and lack of stable secondary structure. Such proteins have been implicated in desiccation tolerance and at least five were found. The majority of the genes identified was confirmed by real-time quantitative PCR to be capable of upregulation in response to evaporative water loss. Remarkably, further database and phylogenetic analysis highlighted four ESTs that are present in the *A. ricciae* genome but which represent genes probably arising from fungi or bacteria by horizontal gene transfer. Therefore, not only can bdelloid rotifers accumulate foreign genes and render them transcriptionally competent, but their expression pattern can be modified for participation in the desiccation stress response, and is presumably adaptive in this context.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/214/1/59/DC1

Key words: bdelloid rotifer, desiccation tolerance, anhydrobiosis, desiccome, LEA protein, hydrophilin, horizontal gene transfer, lateral gene transfer.

INTRODUCTION

Certain organisms are able to survive almost complete loss of their internal water, entering a state of suspended animation called anhydrobiosis. When water becomes available again, the organism revives and resumes its normal activities. These organisms have been called ‘sleeping beauties’ since they appear not to age during dormancy (Hengherr et al., 2008; Ricci and Covino, 2005). Desiccation tolerance is relatively widespread among prokaryotes and unicellular eukaryotes [e.g. baker’s yeast, *Saccharomyces cerevisiae* (Potts, 1994)], but is less common in multicellular eukaryotes, being exclusive to some life stages of plants (mainly seeds and pollen), a number of resurrection plants, and some invertebrates, with well-known examples among the arthropods, tardigrades, nematodes and bdelloid rotifers (Alpert, 2006; Clegg, 2001).

In some organisms, an increase in levels of di- and oligosaccharides, especially the non-reducing sugars trehalose (in animals) and sucrose (in plants), has been associated with the ability to survive desiccation (Crowe et al., 1998; Hoekstra et al., 2001). For example, the anhydrobiotic larva of the chironomid *Polypedilum vanderplanki* accumulates ~18% trehalose by dry weight as it loses water, and the level of trehalose correlates with variation in survival among individuals (Watanabe et al., 2002). Such correlations, together with the powerful stabilising properties of trehalose *in vitro* (Colaco et al., 1992), have led to models of anhydrobiosis in which non-reducing disaccharides play a central role, and most frequently are proposed to have water replacement or vitrification functions (Crowe et al., 1998; Crowe et al., 1992). However, this is not the whole story (Tunnacliffe and Lapinski, 2003): bdelloid rotifers, for example, contain no trehalose and apparently lack the genes to synthesise it (Caprioli et al., 2004; Lapinski and Tunnacliffe, 2003). In the yeast *S. cerevisiae*, whose desiccation tolerance is well attested, trehalose synthesis can be abolished by mutation with only a modest reduction in survival (Ratnakumar and Tunnacliffe, 2006).

Other molecules must contribute to desiccation tolerance, therefore, and candidates have emerged from studies in several organisms. These include the hydrophilic late embryogenesis abundant (LEA) proteins, molecular chaperones, amphiphiles and antioxidants (Burnell and Tunnacliffe, 2010; Hoekstra et al., 2001; Ingram and Bartels, 1996). The latter category, for example, is likely to be important because desiccation disrupts the function of electron transfer chains, thereby elevating levels of reactive oxygen species (ROS) which have a detrimental effect on cell components (Kranner and Birtic, 2005; Rizzo et al., 2010). Direct genetic evidence of the involvement of antioxidants in anhydrobiosis was obtained recently when knockdown of glutathione peroxidase transcripts in the nematode *Panagrolaimus superbus* was shown to decrease survival after desiccation (Reardon et al., 2010). The LEA proteins were first discovered in plant seeds but were later found in various invertebrates, suggesting a conserved response to desiccation stress

(Tunnacliffe et al., 2010). This diverse class of proteins is characterised by a versatility of function probably relating to their lack of defined structure: they are examples of intrinsically disordered proteins (IDPs) (Tompa, 2009). Despite their disordered nature, IDPs are known to carry out a range of different, and sometimes multiple, functions (Tompa et al., 2005), and LEA proteins are not unusual in this respect. Thus, LEA proteins have been implicated as molecular shields or chaperones, membrane protectants, ion sinks, hydration buffers and antioxidants (Battaglia et al., 2008; Shih et al., 2008; Tunnacliffe et al., 2010; Tunnacliffe and Wise, 2007).

Despite this progress, a more comprehensive approach to determine key adaptations in desiccation tolerance, collectively called the 'desiccome' (Potts, 2004), is required and gene discovery programmes are well advanced in a number of organisms. Thus, experimental approaches that identified genes upregulated by dehydration were first established in the resurrection plants (Bartels, 2005; Illing et al., 2005) and have more recently been implemented in invertebrates, primarily in tardigrades (Mali et al., 2010) and nematodes (Gal et al., 2003; Reardon et al., 2010; Tyson et al., 2007). Studies of this type could be particularly informative in bdelloid rotifers, as the anhydrobiology of these aquatic micro-invertebrates has unusual aspects: besides their lack of non-reducing disaccharides, the bdelloid LEA proteins defined to date are not especially hydrophilic and can exhibit secondary structure in solution (Pouchkina-Stantcheva et al., 2007). Furthermore, the genetics of bdelloid rotifers is also unusual: they are the only well-characterised anciently asexual metazoans, having reproduced exclusively by thelytoky (the production of females parthenogenetically) for at least 35 million years (Mark Welch and Meselson, 2000; Rice and Friberg, 2007), and they show a high frequency of horizontal gene transfer (HGT; also known as lateral gene transfer) in sub-telomeric chromosome regions (Gladyshev et al., 2008). To begin to understand the desiccome of the bdelloid rotifer, *Adineta ricciae* (Segers and Shiel, 2005), we produced an expressed sequence tag (EST) library enriched for genes that are active during desiccation stress and characterised a subset of clones of both recognised and novel sequences. Some sequences in the former category are apparently not of metazoan origin, suggesting that foreign genes have been appropriated for the desiccation stress response. In addition, analysis of ESTs encoding novel proteins reveals a high proportion of new classes of hydrophilic proteins other than LEA proteins, suggesting this feature as an intrinsic element of the bdelloid desiccome.

MATERIALS AND METHODS

Rotifer maintenance

The bdelloid rotifer *Adineta ricciae* Segers and Shiel, 2005 (formerly known as *Adineta* sp. 1) was used as a model to study gene regulation in a desiccation-tolerant organism. Clonal cultures of *A. ricciae* were maintained in plastic bottles or flasks; we used either plastic roller bottles (Corning Life Sciences, Amsterdam, The Netherlands) kept horizontal to maximize surface-to-volume ratio, with about 300 ml of autoclaved ultra high purity (UHP) water, or cell culture flasks (between 35 and 175 cm^2, with filter caps) (Nunc, Roskilde, Denmark) with 12–200 ml of the same water. Rotifers were kept at 22°C and fed once every 1–3 days with RAGO (Rotifer and Artemia GrowOut; www.aquaculturesupplies.co.uk: 15 $g l^{-1}$ stock solution prepared in water, autoclaved and allowed to cool and sediment; the supernatant was used for feeding) or *Escherichia coli* [grown in Luria broth (LB) medium overnight, recovered by centrifugation and resuspended in autoclaved UHP water]. Both feedstocks were stored frozen and kept at 4°C for several days during use.

Rotifer desiccation

A. ricciae has previously been shown to survive desiccation at rates of up to ~80% (Ricci et al., 2004; Ricci and Covino, 2005) and similar survival rates are observed in our hands, depending on the drying protocol used. Rotifers to be desiccated were collected by filtration: the container was shaken a few times to detach rotifers, then the rotifer suspension was filtered through 20 μm or 5 μm Nitex filters (Sefar, Heiden, Switzerland). Animals collected on filters were desiccated according to one of three different drying protocols (Ricci et al., 2003). For the first two methods, an environmental test chamber (Temperature Applied Sciences Ltd., Goring, UK) was used, where temperature and relative humidity (RH) can be controlled. In the first protocol, Ricci B, RH is reduced from 98% to 40% over 15 h, then maintained at 40% RH for another 153 h; in the second protocol, Ricci C, RH is maintained at 98% RH for 72 h. In the third protocol, which was used for absolute mRNA quantification of six genes, the filter was placed between two Whatman papers soaked in 1 ml of autoclaved UHP water and left sealed at room temperature for 24 h (RH ~100%); the Petri dish was then opened and left for 48 h until the filter had equilibrated with ambient RH (~33% RH). Control, non-dried rotifers were also collected on filters but RNA was extracted immediately.

RNA extraction

RNA was extracted using TRIzol reagent (Invitrogen, Paisley, UK) according to manufacturer's protocol, washed in ethanol and resuspended in diethylpyrocarbonate-treated water. RNA concentration was measured with a NanoDrop (ND-1000) spectrophotometer (Thermo Fisher Scientific, Loughborough, UK) and quality assessed by analysing the absorbance spectra and the *A*260/280 and *A*260/230 ratios.

EST library construction

A Super SMART^{TM} PCR cDNA Synthesis Kit was used for synthesis of primary cDNA. A cDNA subtraction library was produced with the PCR-select cDNA subtraction kit (both from Clontech-Takara Bio Europe, Saint-Germain-en-Laye, France), following manufacturer's instructions. Briefly, rotifers were collected on filters and RNA was extracted either immediately (control sample, 0 h desiccation), or after dehydration for 24 h at 98% RH. RNA was extracted from both samples; cDNA synthesis was performed for both driver (non-desiccated) and tester (desiccated) sample; concentration was estimated by absorbance at 260 nm; and integrity was confirmed by gel electrophoresis. cDNA was amplified by long distance PCR, purified with phenol–chloroform, digested with *Rsa*I, re-purified and diluted to a final concentration of 280 ng μl^{-1}. Adaptor ligation was followed by hybridisation and selective amplification of differentially expressed fragments, which were then subcloned into pCRII-TOPO vector using a TOPO TA Cloning Kit Dual Promoter (Invitrogen) and transformed into competent *E. coli* cells. Bacteria were grown overnight at 37°C in LB broth and 50 μg ml^{-1} ampicillin, and plasmid DNA extracted with a plasmid mini- or midi-prep kit (Qiagen, Crawley, UK), and checked by restriction digestion. ESTs were sequenced by the dideoxy method at the University of Cambridge Department of Biochemistry Sequencing Facility.

Bioinformatics and sequence analysis

DNA sequences from the subtraction library were analysed with various software packages: 4Peaks (version 1.7.2, www.mekentosj.com), ApE (A plasmid Editor, version 1.17, http://www.biology.utah.edu/jorgensen/wayned/ape) and Geneious

Pro (version 4.8.4, www.geneious.com). Failed sequences (e.g. sequences with indistinguishable peaks, or with multiple/overlapping readings) were discarded at this stage and approved sequences were analysed with blastx (Altschul et al., 1990) (blastx versions 2.2.21–2.2.23, non-redundant protein database). Further analysis on selected ESTs was carried out with tblastx.

Sequences with an E-value lower than E–05 in the blastx search were considered to be defined hits whereas sequences with an E-value higher than E–05 were considered novel. In the first case, sequences were assigned to one of 10 functional categories. Where no match was found with blastx, the six possible open reading frames (ORFs) were obtained with Geneious Pro and the longest was further analysed. Physico-chemical characteristics were inferred with different software packages. The presence of a spliced leader, found at the 5′ end of a proportion of bdelloid mRNAs (Pouchkina-Stantcheva and Tunnacliffe, 2005), and poly(A) tail sequences was determined with Geneious; hydrophilicity was evaluated using ExPASy ProtScale (http://www.expasy.ch/cgi-bin/protscale.pl); theoretical pI, number of charged residues, GRAVY index (grand average hydropathy) and glycine content were calculated with the ExPASy ProtParam tool (http://www.expasy.ch/tools/protparam.html); disorder predictions were performed at PONDR (Predictors of Natural Disordered Regions; http://www.pondr.com) using Uversky plots, VL-TX and VL3 algorithms (Li et al., 1999; Radivojac et al., 2003), and with FoldIndex (Prilusky et al., 2005) (http://bip.weizmann.ac.il/fldbin/findex).

Phylogenetic analysis

To support the foreign origin of some ESTs, phylogenetic analyses were performed using Geneious. ORFs were analysed with blastp and amino acid sequences corresponding to the ten best species matches were downloaded and aligned with ClustalW, and a Neighbour-Joining tree was built (Jukes-Cantor genetic distance model; without designating any outgroup *a priori*). Bootstrap support was calculated with 1000 iterations, and taxa were colour-coded in the consensus tree. Sequences were eliminated where long branches caused all other branches to collapse.

Quantitative PCR

Gene expression profile before and after dehydration was usually studied by relative real-time quantitative PCR (qPCR), normalising gene expression against a reference gene whose expression level was expected to remain constant, or nearly so (either actin or 18S or both). Template cDNA was obtained as described above and all PCR run in a RotorGene 3000 using the SYBR-Green PCR kit (Qiagen). In a number of cases, absolute quantification was performed with the same instrument using the Real-Time RT-qPCR kit (Qiagen), as described previously (Browne et al., 2004). In this case, plasmid DNA from bacterial stocks was extracted with a midi-prep kit (Qiagen), quantified by ultraviolet absorbance with a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific), *in vitro* transcribed with a MEGAscript Kit (Applied Biosystems/Ambion, Warrington, UK) according to manufacturer's instructions using either T7 or SP6 polymerase depending on fragment orientation, and the total number of target molecules quantified by serial dilution (generally in the range of $0.001–1000\,ng\,\mu l^{-1}$).

RESULTS

An EST library of dehydration-responsive genes

Bdelloid rotifers require a slow rate of drying for maximum survival (Lapinski and Tunnacliffe, 2003; Ricci et al., 2003), and we therefore reasoned that a number of metabolic adaptations need to be activated for successful anhydrobiosis in these invertebrates. To attempt to identify adaptations regulated at the transcriptional level, we generated an EST library enriched for sequences that are over-represented in the bdelloid transcriptome after 24 h desiccation compared to non-dried controls. The initial 93 readable sequences were further analysed for possible duplicates, returning a total of 75 unique putative candidates that were then compared with known sequences using the BLAST search tool. Blastx returned 36 sequences with a significant match in the databases (listed in Table 1), and implicated the remaining 39 ESTs as novel sequences (nearest match scoring >E–05). Of these, eight ESTs (F24-15, F24-19, F24-26, F24-31, F24-38, F24-54, F24-91 and F24-98; accession numbers HO188837, HO188839, HO188841, HO188843, HO188845, HO188853, HO188864 and HO188866, respectively) did not show any clear ORFs (≥50 amino acids), and were therefore excluded from further analyses; the remaining 31 ESTs are listed in Table 2. Three of the sequences without clear ORFs (F24-15, F24-38 and F24-98) were included in expression profile studies (see below).

ESTs with database matches include foreign genes

The 36 sequences with a significant match in protein databases can be placed in various categories as shown in Table 1. The largest two categories are metabolism and stress, consistent with the need to reprogramme cell biochemistry and to launch damage prevention and repair systems in the face of desiccation stress. The former category is diverse, although several examples relate to carbohydrate metabolism. In the latter category, more than half the ESTs (F24-6, F24-30, F24-61b, F24-74) represent genes concerned with oxidative stress, in line with the importance of redox balancing and ROS management during desiccation. Two further sequences are implicated in DNA repair, an essential function after the DNA damage resulting from desiccation (Mattimore and Battista, 1996). F24-37 identifies an E2 ubiquitin-conjugating enzyme (RAD6) required for lesion repair, and F24-119 represents a DNA polymerase λ, involved in double strand break repair. An LEA protein sequence is also found in the stress category, and this corresponds to one of the pair of very similar LEA protein genes previously described for *A. ricciae* (Pouchkina-Stantcheva et al., 2007). Other categories with small numbers of ESTs are protein homeostasis, cell structure, RNA processing, apoptosis, ribosomal proteins, signalling, transport and translation (Table 1). In the cell structure category, four actin ESTs were found with a few differences in the nucleotide and amino acid sequences (supplementary material Fig. S1); where actin was used as a reference gene in quantitative PCR experiments, the sequence corresponding to F24-59 was used.

Included in Table 1 for each bdelloid EST is the species giving the best blastx match against the set of known protein sequences. Intriguingly, six of these, shown by grey shading in Table 1, gave closest matches to non-metazoan species. Two more sequences, F24-11 and F24-86, also gave a non-metazoan species as a top hit, but the E-values were relatively high and therefore these ESTs were not analysed further. Since bdelloid rotifers have been shown recently to accumulate foreign DNA sequences from bacterial, fungal and plant sources on a large scale, at least some of which are known to be expressed (Gladyshev et al., 2008), an additional test was performed to examine the evolutionary origins of these six sequences. A second BLAST algorithm, tblastx, which translates each EST and compares potential ORFs against a database of translated non-redundant nucleotide sequences, was used, and separate lists were compiled of best hits against targets among the

Table 1. Expressed sequence tags (ESTs) from *A. ricciae* with significant database matches; in 10 functional categories

Clone ID	Accession number	bp*	Best blastx hits†	Species‡	E-value
Metabolism					
F24-2	HO188794	383	Glucose-repressible gene grg1	*Podospora anserina*	2E–10
F24-10	HO188795	561^{A}	Glucosidase, maltase-glucoamylase	*Nasonia vitripennis*	1E–17
F24-11	HO188796	610	Amidase; glutamyl-tRNA(Gln) amidotransferase subunit A	*Flavobacteria bacterium* BAL38	4E–07
F24-14	HO188797	606	Branched chain keto acid dehydrogenase	*Sus scrofa*	5E–78
F24-16	HO188798	399	Amidase	alpha proteobacterium BAL199	1E–40
F24-20	HO188799	383	Putative major royal jelly protein; gluconolactonase	*Methylobacterium*	5E–30
F24-45	HO188800	416^{A}	Phosphatidylinositol-4-phosphate 5-kinase (PIPKc)	*Ixodes scapularis*	2E–10
F24-64	HO188801	590^{A}	ebna2 binding protein	*Culex quinquefasciatus*	1E–14
F24-69	HO188802	374	Countin-like protein	*Philodina roseola*	3E–14
F24-110	HO188803	539	Carbamoyl phosphate synthetase	*Acyrthosiphon pisum*	3E–66
Stress					
F24-6	HO188804	633	Catalase	*Anopheles gambiae*	3E–65
F24-22	HO188805	795^{A}	ArLEA1A	*Adineta ricciae*	9E–75
F24-30	HO188806	566	Similar to AT26381p, Fe-S cluster biosynthesis	*Nasonia vitripennis*	2E–45
F24-37	HO188807	653^{A}	Ubiquitin-conjugating enzyme E2A/RAD6 homologue	*Caligus rogercresseyi*	3E–38
F24-61b	HO188808	160	Superoxide dismutase	*Bos taurus*	4E–08
F24-74	HO188809	739	Dioxygenase; Iron/ascorbate oxidoreductase/dioxygenase family protein	*Candida albicans* SC5314	7E–33
F24-119	HO188810	447	Polymerase (DNA directed), lambda	*Xenopus* (*Silurana*) *tropicalis*	1E–41
Protein homeostasis					
F24-8	HO188811	439	IBR (In Between Ring fingers) domain	*Entamoeba histolytica* HM-1:IMSS	7E–33
F24-33	HO188812	565	Similar to DnaJ (Hsp40) homolog	*Apis mellifera*	3E–26
F24-35	HO188813	410	Similar to LON peptidase N-terminal domain and ring finger 1	*Danio rerio*	1E–14
F24-50	HO188814	412	Ubiquitin-like/S30 ribosomal fusion protein	*Equus caballus*	2E–19
Cell structure					
F24-40	HO188815	472	Similar to coiled-coil domain containing 87	*Strongylocentrotus purpuratus*	6E–11
F24-48	HO188816	591	Cadherin 1	*Gallus gallus*	2E–18
F24-59	HO188817	469	Actin	*Onychoteuthis compacta*	2E–80
F24-62	HO188818	719			
F24-68	HO188819	367			
F24-78	HO188820	469			
RNA processing					
F24-75	HO188821	646	DDX47 RNA helicase	*Mus musculus*	5E–58
F24-112	HO188822	564	DiGeorge syndrome critical region gene 8 (miRNA maturation)	*Mus musculus*	8E–10
F24-113	HO188823	566	RNA recognition motif (RRM)	*Ixodes scapularis*	8E–26
Apoptosis					
F24-25	HO188824	600	Programmed cell death 4	*Danio rerio*	3E–23
F24-108	HO188825	608	BIR: baculoviral inhibition of apoptosis (IAPs) protein repeat domain	*Ornithorhynchus anatinus*	2E–17
Ribosomal					
F24-28	HO188826	760_{SL}	60S ribosomal protein L22	*Salmo salar*	4E–38
F24-87	HO188827	627	Similar to ribosomal L1 domain-containing protein 1	*Macaca mulatta*	3E–18
Signalling					
F24-63	HO188828	560	Tyrosine phosphatase	*Ciona intestinalis*	7E–17
F24-86	HO188829	324	Calcium-binding EF-hand domain-containing protein	*Dictyostelium discoideum* AX4	4E–06
Transport					
F24-3	HO188830	665	High-affinity inorganic phosphate transporter	*Glomus intraradices*	3E–28
F24-5	HO188831	314	Plasma membrane calcium ATPase	*Adineta vaga*	6E–32
Translation					
F24-103	HO188832	249	Translation initiation factor eIF-2B subunit alpha	*Salmo salar*	9E–23

bp, base pairs.

*Nucleotide length; if a poly(A) tail is present, this is shown by (^{A}) but is not included in the length value. Subscript SL indicates the presence of a spliced leader.

†The likely identity of the respective gene.

‡The organism with the best match.

E-values are all <1E–05. Grey shading is used for those ESTs that are candidate foreign sequences and that were subjected to further analysis (see Table 3).

Table 2. Analysis of open reading frames (ORFs) from expressed sequence tags (ESTs) without significant database matches by blastx analysis; i.e. 'novel' sequences

Clone ID	Accession no.	ORF (aa)	GRAVY	VL-TX	VL3	FoldIndex unfoldability	Uversky	pI	aa charge % –	aa charge % +	% Gly
BSA	NM180992	617	–0.43	0.21	0.35	0.09	x	5.83	16.0	13.9	2.9
ArLEA1A	EF554866	429	**–0.47**	0.23	**0.60**	0.08	x	5.44	18.4	16.8	5.4
ArLEA1B	EF554865	384	**–0.49**	0.34	**0.60**	0.07	x	5.42	18.2	16.7	5.2
AavLEA1	AF423069	143	**–1.58**	**0.64**	**0.77**	**–0.28**	✓	5.34	21.0	18.2	9.1
Anhydrin	AY340998	86	**–1.56**	**0.80**	**0.87**	**–0.32**	✓	10.06	14.0	22.1	2.3
F24-4	HO188833	251	0.40	0.31	0.27	0.37	x	7.23	4.0	4.0	13.9
F24-7	HO188834	91 [A]	–0.53	0.34	0.38	–0.01	x	4.77	13.2	4.4	11.0
F24-12	HO188835	75 [A]	**–0.70**	**0.60**	**0.60**	**–0.17**	✓	11.54	2.7	22.7	0.0
F24-13	HO188836	258	–0.48	0.46	0.52	0.07	x	5.53	13.2	10.5	1.2
F24-17a	HO188838	120	–0.13	0.05	0.11	0.14	x	9.70	5.8	11.7	1.7
F24-21	HO188840	123	0.59	0.21	0.20	0.40	x	5.13	8.9	6.5	10.6
F24-27	HO188842	136 [A]	–0.33	0.47	0.51	0.12	x	6.03	11.8	10.3	2.2
F24-36	HO188844	140	0.05	0.16	0.43	0.24	x	8.15	6.4	8.6	5.0
F24-39	HO188846	207	**–0.84**	**0.70**	**0.58**	**–0.05**	✓	10.34	2.9	5.8	4.8
F24-43b	HO188847	84	0.42	0.09	0.10	0.33	x	8.64	4.8	8.3	0.0
F24-44	HO188848	235	–0.51	0.17	0.16	0.06	x	5.67	13.6	11.1	4.3
F24-47	HO188849	106	**–0.97**	**0.57**	**0.70**	**–0.17**	✓	4.23	16.0	4.7	0.9
F24-49	HO188850	126 [SL]	**–0.65**	0.30	0.35	**–0.04**	✓	9.77	7.9	16.7	2.4
F24-51	HO188851	112	–0.57	0.20	0.36	0.05	x	7.87	13.4	14.3	0.9
F24-52b	HO188852	66	–0.03	0.35	0.48	0.23	x	7.07	7.6	7.6	9.1
F24-55	HO188854	77	–0.56	0.04	0.27	0.07	x	6.75	6.5	6.5	7.8
F24-56	HO188855	57 [A]	–0.40	0.22	0.33	0.05	x	4.45	14.0	7.0	3.5
F24-57	HO188856	203	0.82	0.28	0.34	0.49	x	7.68	5.9	6.4	11.3
F24-58	HO188857	109 [SL]	–0.62	0.23	0.31	0.01	x	5.71	12.5	8.0	2.7
F24-66	HO188858	178	**–0.67**	**0.59**	**0.57**	**0.00**	x	5.22	16.9	13.5	3.9
F24-76	HO188859	101	–0.56	0.30	0.21	0.05	x	5.88	13.9	11.9	5.0
F24-77	HO188860	173	0.66	0.19	0.19	0.44	x	6.70	3.5	3.5	13.9
F24-82	HO188861	183	**–0.49**	**0.67**	**0.59**	0.08	x	9.40	3.8	4.9	1.1
F24-85	HO188862	176	–0.56	0.31	0.33	0.02	x	4.80	14.2	9.1	1.1
F24-90	HO188863	180	–0.03	0.18	0.15	0.20	x	5.74	10.0	7.2	1.7
F24-97	HO188865	193	0.57	0.18	0.18	0.41	x	8.99	3.1	4.1	13.0
F24-104	HO188867	151	**–0.63**	0.38	**0.64**	**–0.05**	✓	11.14	7.9	17.2	2.0
F24-105	HO188868	101	–0.12	0.16	0.21	0.13	x	9.39	8.0	14.9	1.1
F24-106	HO188869	198	**–0.53**	**0.51**	**0.53**	0.08	x	7.21	8.1	8.1	3.0
F24-107	HO188870	120	0.18	0.19	0.08	0.22	x	9.54	5.8	13.3	3.3
F24-120	HO188871	232	0.47	0.30	0.26	0.39	x	7.19	3.9	3.9	13.4

BSA (bovine serum albumin), ArLEA1A, ArLEA1B, AavLEA1 and anhydrin are included for comparison.

Superscript A indicates the presence of a poly(A) tail; superscript SL indicates the presence of a spliced leader.

Hydrophilicity is indicated by a negative GRAVY index (grand average hydropathy); VL-TX and VL3 and FoldIndex unfoldability are different measures of disorder; a tick under Uversky indicates that the protein sequence maps in unfolded space in a Uversky plot of mean hydropathy against mean net charge.

Hydrophilic candidate intrinsically disordered proteins are shaded light grey if they match two out of four criteria for disorder, or dark grey if they match three out of four criteria (see text for details).

Bold type indicates the parameters that show, for the highlighted ESTs, higher hydrophilicity than BSA, a score of >0.5 for VL-TX and VL3, and a FoldIndex score of ≤0.

pI, isoelectric point.

metazoa, plants, fungi, 'other' (anything excluding metazoans, plants, fungi, eubacteria, viruses and Archea) and eubacteria (Table 3). Two of the six ESTs were found to give matches against metazoans: F24-74 returned an E value of 4E–11 for a predicted protein from *Saccoglossus kowalevskii*, a hemichordate, and a second hit for *Drosophila sechellia* (1E–07), while F24-8 returned an E-value of 3E–16 against a sequence from a hypothetical protein from *Branchiostoma floridae*, a cephalochordate, a second hit for the stony coral *Acropora millepora*, *via* a Total Shotgun Approach (4E–16), and a subsequent hit for the human body louse (1E–15). Therefore, although the ESTs F24-74 and F24-8 gave considerably lower tblastx E-values against non-metazoans, there was sufficient doubt to eliminate them from further consideration.

For the remaining four ESTs, however, the tblastx results supported an exogenous origin for the corresponding genes. F24-2, whose sequence shows similarity to glucose-repressible genes of unknown function, gave no hits with an E-value <1 among the metazoa, but confirmed *Podospora anserina* as the best match (4E–14) among the fungi. Hits were also obtained among the plants, with *Oryza rufipogon* at 6E–09, but no bacterial or protist sequences with significant matches were returned. F24-3, which probably represents a phosphate transporter gene, gave a top tblastx match of 2E–43 against a sequence from *Ustilago maydis*, a fungal plant pathogen, whereas the best metazoan example was a predicted sequence from honey bee (*Apis mellifera*), which returned a not-significant E-value of 0.008. Blastx for F24-16 identified a dioxygenase gene, scoring best against an alpha proteobacterium, and tblastx also gave the most significant hit against a bacterium, and a slightly less significant hit for a green alga and a protist, without returning any significant hit among metazoans. Finally, in

Table 3. Expressed sequence tags from *A. ricciae* genes potentially arising from horizontal gene transfer

Functional category	Clone ID	blastx best hit	tblastx best hit: Metazoa (taxid: 33208)	Plants (taxid: 3193)	Fungi (taxid: 4751)	Other	Bacteria (taxid: 2)
Metabolism	F24-2	*Podospora anserina* 2E–10	*Homo sapiens* 4.0	*Oryza rufipogon* 6E–09	*Podospora anserina* 4E–14	*Paramecium tetraurelia* 7.3	*Zunongwangia profunda* 0.03
	F24-16	alpha proteobacterium BAL199 1E–40	*Lepeophtheirus salmonis* 0.03	*Chlamydomonas reinhardtii* 6E–32	*Penicillium marneffei* 2E–08	*Perkinsus marinus* 1E–24	*Ochrobactrum anthropi* 2E–48
	F24-20	*Methylobacterium chloromethanicum* CM4 5E–30	*Homo sapiens* 0.9	*Oryza sativa* 8.1	*Aspergillus niger* 3E–04	*Trypanosoma cruzi* 5.3	*Methylobacterium radiotolerans* 2E–32
Stress	F24-74	*Candida albicans* SC5314 7E–33	*Saccoglossus kowalevskii* 4E–11 *Drosophila sechellia* 1E–07	*Picea glauca* 3E–12	*Pichia pastoris* 2E–27	*Naegleria gruberi* predicted protein 8E–11 *Phaeodacxtylum tricornutum* CCAP 1055/1 1E–08	*Thimonas* sp. Str. 3As 5E–09
Protein homeostasis	F24-8	*Entamoeba histolytica* HM–1:IMSS 7E–33	*Branchiostoma floridae* hypothetical protein 3E–16 *Acropora millepora* 4E–16 *Pediculus humanus corporis* 1E–15	*Arabidopsis thaliana* 1E–16	*Phaeosphaeria nodorum* 2E–34	*Phytophtora capsici* clone CBOT105–I17 7E–17	*Thermicola* sp. JR 0.7
Transport	F24-3	*Glomus intraradices* 3E–28	*Apis mellifera* 0.01	*Populus thicocarpa* 1E–27	*Ustilago maydis* 2E–43	*Paramecium tetraurelia* hypothetical protein 8E–33	*Alicyclobacillus acidocarldarius* 1E–21

Each sequence is classified according to one of the 10 functional categories of Table 1. The clone identification, best hit and E-value after blastx analysis (Table 1), and best hit and E-value after blastx analysis for each taxon (metazoa, plants, fungi, other and bacteria), are listed. Grey shading indicates indicates sequences that are the best candidates for horizontal gene transfer.

Table 1, the best blastx match for F24-20 was with *Methylobacterium chloromethanicum* CM4; the tblastx algorithm also returned a *Methylobacterium* species, albeit different, as the top hit with an E-value of 2E–32. The best-matching metazoan sequence was from human with an E-value of just 0.87, and a fungal sequence gave a marginally significant hit (3E–04). To further confirm the non-metazoan nature of these four sequences, phylogenetic analyses were carried out, confirming a bacterial origin for F24-20 (Fig. 1) and F24-16 (supplementary material Fig. S2A), and a fungal origin for F24-3 (supplementary material Fig. S2B). F24-2 did not produce any matches with metazoa (supplementary material Fig. S2C). In summary, therefore, at least four ESTs in our panel seem to represent genes in the bdelloid rotifer genome that derive from horizontal gene transfer.

Novel ESTs

For the 31 of the 39 novel sequences in the EST library with an identifiable ORF, we used bioinformatics tools to provide insight into some of the potential physico-chemical properties of their predicted proteins. In particular, we were interested in those proteins that might play an analogous role to LEA proteins in other species, since the bdelloid LEA proteins characterised to date seem atypical: they are not particularly hydrophilic, and one of them has secondary structure in the hydrated state (Pouchkina-Stantcheva et al., 2007) (Table 2). We therefore looked for examples of predicted proteins which were both hydrophilic and potentially disordered, assessing the former property using the GRAVY index and the latter with three different algorithms, VL-TX, VL3 and FoldIndex, and a Uversky plot of mean net hydropathy against mean net charge (Table 2). We also calculated the theoretical isoelectric point (pI) for each protein, the percentage of charged amino acids and the glycine content. For comparison, the same analysis was performed for BSA (bovine serum albumin), as a typical soluble globular protein; for the two previously-reported LEA proteins from *A. ricciae* (ArLEA1A and ArLEA1B) (Pouchkina-Stantcheva et al., 2007); and for two more typical hydrophilic IDPs from the anhydrobiotic nematode *Aphelenchus avenae*: AavLEA1, a group 3 LEA protein (Browne et al., 2002; Goyal et al., 2003), and anhydrin, a basic IDP (Browne et al., 2004; Chakrabortee et al., 2010).

As criteria for identifying hydrophilic IDPs, we asked for a GRAVY score more negative than that of BSA (–0.43), and two out of four of the following: VL-TX and VL3 indices higher than 0.5 (i.e. at least 50% disordered), a FoldIndex score less than zero (indicative of proteins that are likely to be unfolded), and location in disordered space in the Uversky plot. Eight examples (grey shading in Table 2) met these criteria; of these, two (F24-47, F24-66) are acidic (pI<6.0), one is broadly neutral (F24-106), and five (F24-12, F24-39, F24-49, F24-82, F24-104) are basic (pI>9.0). Thus, eight out of 31, or 26%, novel sequences are likely to be hydrophilic IDPs, or at least to contain intrinsically disordered regions (IDRs).

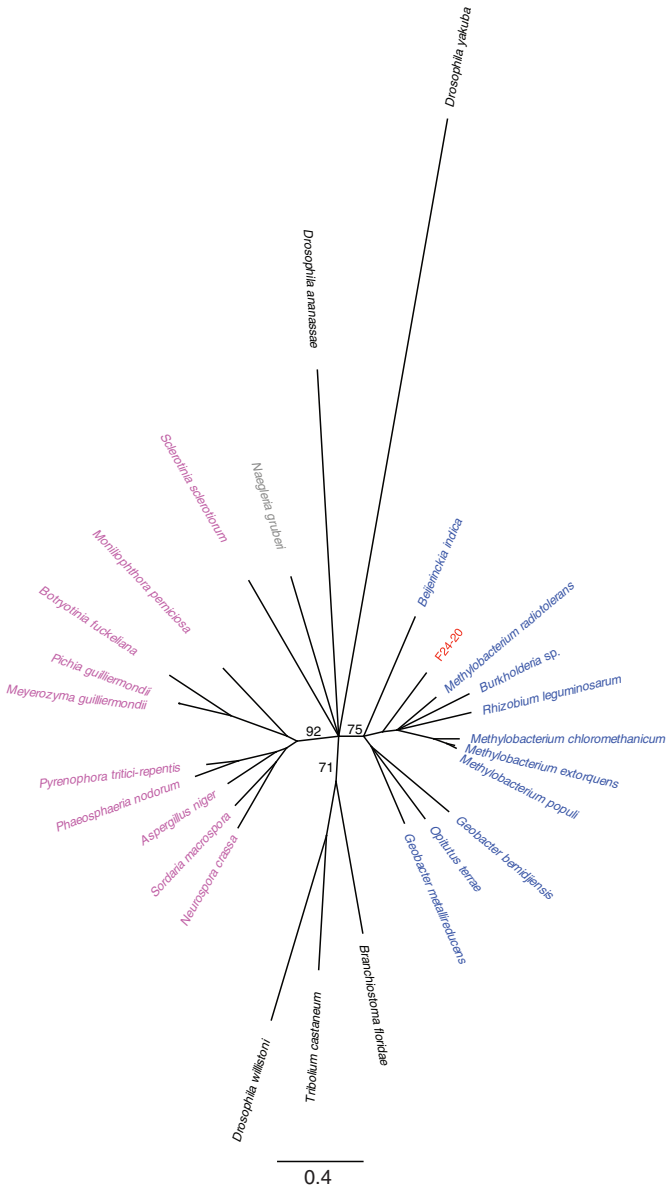

Fig. 1. Phylogenetic tree for F24-20, a putative major royal jelly protein/gluconolactonase. Neighbour-joining consensus tree with bootstrap support after 1000 iterations (bootstrap support is indicated only for the major branches). Taxa are colour-coded: black, metazoans; pink, fungi; blue, bacteria; grey, other. The F24-20 expressed sequence tag is in red. Scale bar indicates number of amino acid substitutions per site.

With a more stringent approach, requiring three out of the four criteria for disorder to be met, three ESTs (F24-49, F24-82, F24-106) were discarded (light grey shading in Table 2), leaving five out of 31, or 16%, as hydrophilic IDPs. Of the EST dataset as a whole (67 ESTs in total, excluding the eight ESTs without a recognisable ORF), hydrophilic IDPs constituted 12% or 7%, respectively, depending on the selection criteria used. This is within the range of values for IDP representation in eukaryote proteomes (Dunker et al., 2000; Tompa, 2009) and therefore suggests that such proteins are not over-represented in the bdelloid rotifer. The IDPs identified do not contain a high proportion of glycine and therefore do not conform to the definition of 'hydrophilins' (Garay-Arroyo et al., 2000), where a minimum of 6% glycine was stipulated.

Gene expression profiles

To confirm the enrichment for dehydration-regulated genes in the EST library, the expression levels of 65 genes were investigated under various drying regimes using qPCR. Three different drying protocols were used, as described in Materials and methods. The results are summarised graphically in Fig. 2 and showed that at least a third of the genes, represented by 21 ESTs, are consistently upregulated, i.e. have more than a twofold increase in mRNA levels, in all the drying experiments. Of these, in some cases the upregulation was independent of the desiccation protocol (e.g. F24-2/grg1, F24-22/ArLEA1A), whereas in other cases a consistently higher expression level was observed when rotifers were subjected to protocol Ricci B (e.g. F24-36/novel, F24-50/ubiquitin-like protein). The latter observation might reflect the more stringent conditions of water loss imposed by this protocol. Another 12 genes showed inconsistent regulation, but were sometimes upregulated by dehydration (e.g. F24-4/novel, F24-45/phosphatidylinositol-4-phosphate 5-kinase). Twenty-six genes were not regulated by water loss, with a fold change in mRNA of between 0.5 and 2 (e.g. F24-11/amidase), and six genes were apparently consistently downregulated (e.g. F24-52b/novel, F24-113/RNA recognition motif). Of particular interest were the four foreign genes identified (see Tables 1 and 2). Most participated to some degree in the response to water loss: one (F24-2/grg1) was consistently upregulated by desiccation in all the experiments performed, two (F24-3/inorganic phosphate transporter and F24-20/putative major royal jelly protein) showed a variable pattern of regulation, and one (F24-16/amidase) was marginally regulated. Similarly, of the hydrophilic IDPs identified (Table 3), the majority (three out of five, using the most stringent selection criteria; see above) were upregulated by evaporative water loss, consistent with a role in desiccation tolerance. In general, 37.5% of the novel ESTs were upregulated and 12.5% were downregulated upon desiccation, while 12.5% were inconsistently regulated.

DISCUSSION

This study demonstrates for the first time that an anhydrobiotic bdelloid rotifer, *A. ricciae*, mounts a significant transcriptional response to evaporative water loss. It therefore seems likely that bdelloids need to make at least a partial adjustment to their biochemistry and physiology through gene regulation to survive desiccation. This is not necessarily expected, because some organisms are apparently capable of anhydrobiosis without the need for metabolic modification (Shannon et al., 2005). Some clues as to the nature of the adjustments occurring in bdelloids were obtained from examination of an EST library enriched for dehydration-regulated gene sequences. Using a subtractive hybridization approach, we identified 75 ESTs that are expressed 24 h after the onset of desiccation, of which 44% (21 defined, 12 novel) are consistently upregulated compared with the fully hydrated animal. A number of other ESTs (12 defined, four novel) represent genes that are upregulated by dehydration under some conditions, giving a total of 65% of the sequences identified that can respond positively to evaporative water loss. Approximately half of all ESTs were identifiable by database matching and their respective genes could be assigned to various broad functional categories, while the remainder had no obvious counterparts in other species and therefore represent novel sequences. Of the recognisable sequences, those in the stress and protein homeostasis categories are likely to be the most relevant to anhydrobiosis, including LEA protein, antioxidant, DNA repair, molecular chaperone and protein clearance genes. Therefore, although we are clearly only sampling a subset of the

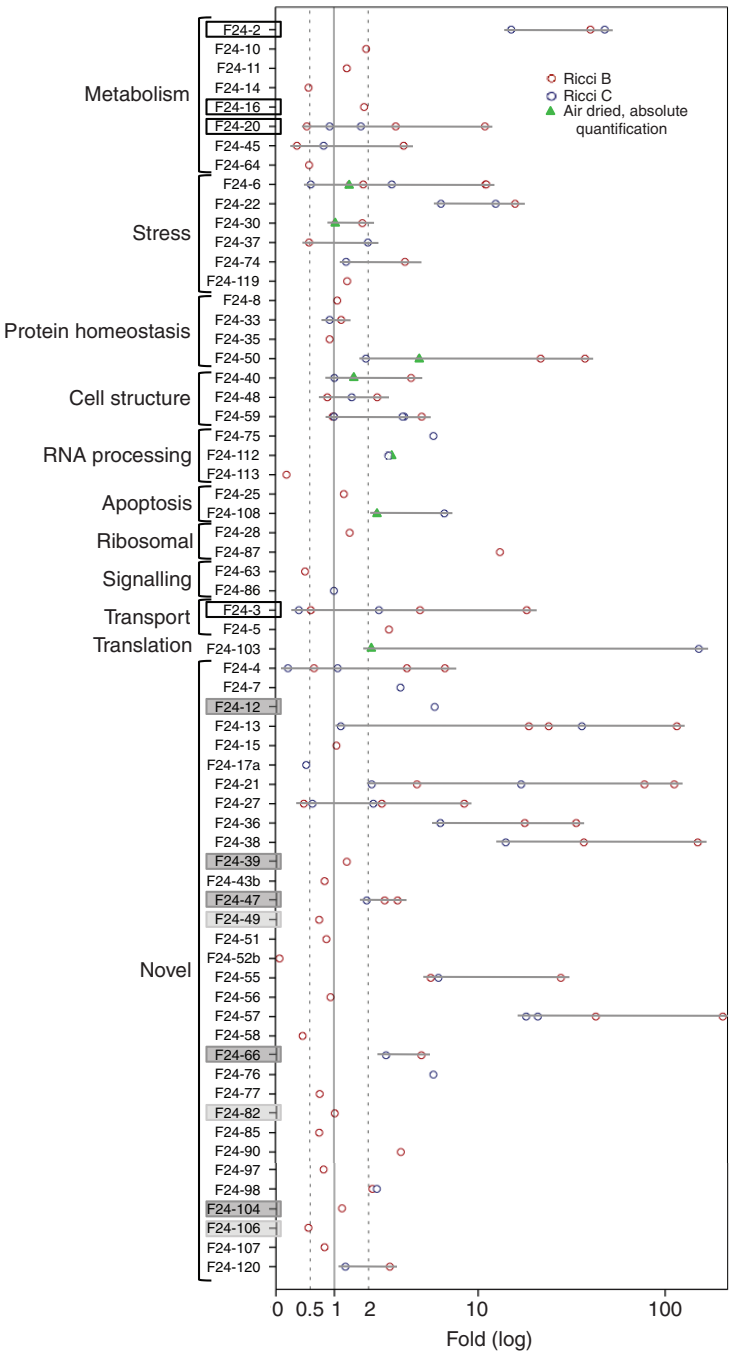

Fig. 2. *A. ricciae* gene regulation under different drying regimes. Expressed sequence tags are listed on the *y*-axis, divided into category groups, and fold regulation is shown on the *x*-axis (log scale). A continuous vertical line indicates no change in transcript level, and flanking dotted lines represent 0.5- and 2-fold regulation, respectively; values within these boundaries are considered to represent no or marginal regulation. Symbols indicate different desiccation protocols (red, Ricci B; blue, Ricci C; green, desiccation in Petri dish – air dried) and the type of qPCR used (circles, relative quantification; triangles, absolute quantification). To facilitate comparison of the data points, different values for the same gene are connected by a horizontal line. ESTs arising from foreign genes are outlined by a clear black square (as listed in Table 3); 'novel' hydrophilic and intrinsically disordered protein ESTs are shaded dark grey or light grey according to the more or less stringent criteria for their identification (see Table 2).

desiccome, bdelloids follow a pattern of gene expression observed in other dehydrating anhydrobiotes, principally nematodes (Adhikari et al., 2009; Gal et al., 2003; Reardon et al., 2010; Tyson et al., 2007) and tardigrades (Mali et al., 2010; Schill et al., 2004). Nevertheless, evidence for the involvement of non-reducing disaccharides in bdelloid rotifers is still lacking.

Only a single LEA protein EST was found in our dataset, corresponding to *Ar-lea-1A*, one of two very similar LEA protein genes described previously in *A. ricciae* (Pouchkina-Stantcheva et al., 2007). Both bdelloid LEA proteins are unusual compared with group 3 LEA proteins reported in other species because they do not exhibit a high degree of disorder; indeed, one of the bdelloid examples, ArLEA1B, adopts some secondary structure in solution, and can be considered a molten globule rather than an IDP proper. Moreover, N-terminal and C-terminal signal sequences suggest that both bdelloid LEA proteins are located in the lumen of the endoplasmic reticulum; preliminary experimental data are consistent with this (C. B. Tripathi and A.T., unpublished). This subcellular localisation for group 3 LEA proteins is unusual, although not unprecedented (Tunnacliffe and Wise, 2007), with most examples being found in the cytoplasm. It might be expected, therefore, that other LEA proteins with different properties are present in *A. ricciae*, particularly since the bdelloid rotifers' relatives, monogonont rotifers, have been shown to possess at least two different LEA proteins, both of which are more typically hydrophilic and probably located in the cytoplasm (Denekamp et al., 2010). Other invertebrates are known to contain several LEA proteins including group 1 sequences (Mali et al., 2010; Sharon et al., 2009), a class

of LEA proteins previously thought to be plant specific. A more detailed analysis of the bdelloid LEA proteome will be possible once the *A. ricciae* genome sequence is available (C.B., A. Carr, A.T. and G. Micklem, unpublished).

The presence of only one type of LEA protein sequence in the EST dataset led us to search for other types of unstructured hydrophilic proteins that might have an analogous role in desiccation tolerance. We did not find an example of a 'hydrophilin' (Garay-Arroyo et al., 2000), i.e. with both high hydrophilicity and high glycine content, but we did uncover candidate hydrophilic IDPs that shared some physico-chemical characteristics with LEA proteins. Of the five strongest candidates, three are upregulated during evaporative water loss and thus participate in the desiccation stress response. In addition, at least 12 other novel sequences are also consistently upregulated by dehydration; as the function of these genes is completely unknown, they represent an interesting opportunity for further studies.

Intriguingly, at least four ESTs, i.e. 11% of the recognisable sequences, are strong candidates for horizontal gene transfer from fungal (F24-3) or bacterial (F24-2, F24-16, F24-20) origins. Moreover, because at least three of the four examples can be upregulated in response to water loss, they are not only capable of being expressed in the bdelloid rotifer, but also form part of the *A. ricciae* desiccome. These ESTs (and indeed all those reported in this paper) are also present in the *A. ricciae* genome sequence and are therefore are not the result of contamination of bdelloid cultures (C.B., A. Carr, G. Micklem and A.T., unpublished). This confirms and extends previous findings (Gladyshev et al., 2008), which demonstrated a remarkably high frequency of foreign genes in the subtelomeric regions of *A. vaga* and *Philodina roseola* chromosomes. Bdelloid rotifers, therefore, seem to be unusual among higher eukaryotes for the extent to which they assimilate foreign genes. For example, where animal genomes have been analysed, only a few cases of individual HGT are known (Keeling and Palmer, 2008). If the whole transcriptome reflects the results described in this study, bdelloids might express hundreds of foreign genes. Presumably this is adaptive, conferring some selective advantage and perhaps partially compensating for the inability of bdelloids, as asexuals, to accumulate advantageous genetic variation through sexual exchange. Gladyshev et al. have speculated that the facility to incorporate foreign DNA into their genomes results from the desiccation tolerance of bdelloids, as the dry state is expected to result in chromosome breakage and cell membrane permeability, both of which are likely to stimulate HGT (Gladyshev et al., 2008). This would rather neatly intertwine the two most remarkable characteristics of bdelloid rotifers, namely anhydrobiosis and asexual reproduction.

LIST OF ABBREVIATIONS

BLAST	Basic Local Alignment Search Tool
EST	expressed sequence tag
GRAVY	grand average hydropathy
HGT	horizontal gene transfer
IDP	intrinsically disordered protein
LEA	late embryogenesis abundant
ORF	open reading frame
PCR	polymerase chain reaction
PONDR	predictor of natural disordered regions
RH	relative humidity
ROS	reactive oxygen species

ACKNOWLEDGEMENTS

We thank Prof. Tim Barraclough (Imperial College, London) and Dr Esther Lubzens (Israel Oceanographic and Limnological Research) for helpful comments on the manuscript. This work was funded by Biotechnology and Biological Sciences Research Council (UK) grants 8/S19912 and BB/F020856/1, and European Research Council Advanced Investigator grant 233232. Access to PONDR® was provided by Molecular Kinetics (6201 La Pas Trail–Ste 160, Indianapolis, IN 46268; 317-280-8737; main@molecularkinetics.com). VL-XT is copyright ©1999 by the WSU Research Foundation, all rights reserved. PONDR® is copyright ©2004 by Molecular Kinetics, all rights reserved.

REFERENCES

Adhikari, B. N., Wall, D. H. and Adams, B. J. (2009). Desiccation survival in an Antarctic nematode: molecular analysis using expressed sequenced tags. *BMC Genomics* **10**, 69.

Alpert, P. (2006). Constraints of tolerance: why are desiccation-tolerant organisms so small or rare? *J. Exp. Biol.* **209**, 1575-1584.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. and Lipman, D. J. (1990). Basic local alignment search tool. *J. Mol. Biol.* **215**, 403-410.

Bartels, D. (2005). Desiccation tolerance studied in the resurrection plant *Craterostigma plantagineum*. *Integr. Comp. Biol.* **45**, 696-701.

Battaglia, M., Olvera-Carrillo, Y., Garciarrubio, A., Campos, F. and Covarrubias, A. A. (2008). The enigmatic LEA proteins and other hydrophilins. *Plant Physiol.* **148**, 6-24.

Browne, J., Tunnacliffe, A. and Burnell, A. (2002). Anhydrobiosis-plant desiccation gene found in a nematode. *Nature* **416**, 38-38.

Browne, J. A., Dolan, K. M., Tyson, T., Goyal, K., Tunnacliffe, A. and Burnell, A. M. (2004). Dehydration-specific induction of hydrophilic protein genes in the anhydrobiotic nematode *Aphelenchus avenae*. *Eukaryotic Cell* **3**, 966-975.

Burnell, A. M. and Tunnacliffe, A. (2011). Gene induction and desiccation stress in nematodes. In *Molecular and Physiological Basis of Nematode Survival* (ed. R. N. Perry and D. Wharton). Wallingford, UK: CABI Publishing.

Caprioli, M., Krabbe Katholm, A., Melone, G., Ramløv, H., Ricci, C. and Santo, N. (2004). Trehalose in desiccated rotifers: a comparison between a bdelloid and a monogonont species. *Comp. Biochem. Physiol.* **139A**, 527-532.

Chakrabortee, S., Meersman, F., Kaminski Schierle, G. S., Bertoncini, C. W., McGee, B., Kaminski, C. F. and Tunnacliffe, A. (2010). Catalytic and chaperone-like functions in an intrinsically disordered protein associated with desiccation tolerance. *Proc. Natl. Acad. Sci. USA* **107**, 16084-16089.

Clegg, J. S. (2001). Cryptobiosis-a peculiar state of biological organization. *Comp. Biochem. Physiol.* **128B**, 613-624.

Colaco, C., Sen, S., Thangavelu, M., Pinder, S. and Roser, B. (1992). Extraordinary stability of enzymes dried in trehalose: simplified molecular biology. *Biotechnology* **10**, 1007-1011.

Crowe, J. H., Hoekstra, F. A. and Crowe, L. M. (1992). Anhydrobiosis. *Annu. Rev. Physiol.* **54**, 579-599.

Crowe, J. H., Carpenter, J. F. and Crowe, L. M. (1998). The role of vitrification in anhydrobiosis. *Annu. Rev. Physiol.* **60**, 73-103.

Denekamp, N. Y., Reinhardt, R., Kube, M. and Lubzens, E. (2010). Late embryogenesis abundant (LEA) proteins in nondesiccated, encysted, and diapausing embryos of rotifers. *Biol. Reprod.* **82**, 714-724.

Dunker, A. K., Obradovic, Z., Romero, P., Garner, E. C. and Brown, C. J. (2000). Intrinsic protein disorder in complete genomes. *Genome Inform. Ser. Workshop Genome Inform.* **11**, 161-171.

Gal, T. Z., Glazer, I. and Koltai, H. (2003). Differential gene expression during desiccation stress in the insect-killing nematode *Steinernema feltiae* IS-6. *J. Parasitol.* **89**, 761-766.

Garay-Arroyo, A., Colmenero-Flores, J. M., Garciarrubio, A. and Covarrubias, A. A. (2000). Highly hydrophilic proteins in prokaryotes and eukaryotes are common during conditions of water deficit. *J. Biol. Chem.* **275**, 5668-5674.

Gladyshev, E. A., Meselson, M. and Arkhipova, I. R. (2008). Massive horizontal gene transfer in bdelloid rotifers. *Science* **320**, 1210-1213.

Goyal, K., Tisi, L., Basran, A., Browne, J., Burnell, A., Zurdo, J. and Tunnacliffe, A. (2003). Transition from natively unfolded to folded state induced by desiccation in an anhydrobiotic nematode protein. *J. Biol. Chem.* **278**, 12977-12984.

Hengherr, S., Brummer, F. and Schill, R. O. (2008). Anhydrobiosis in tardigrades and its effects on longevity traits. *J. Zool.* **275**, 216-220.

Hoekstra, F. A., Golovina, E. A. and Buitink, J. (2001). Mechanisms of plant desiccation tolerance. *Trends Plant Sci.* **6**, 431-438.

Illing, N., Denby, K. J., Collett, H., Shen, A. and Farrant, J. M. (2005). The signature of seeds in resurrection plants: a molecular and physiological comparison of desiccation tolerance in seeds and vegetative tissues. *Integr. Comp. Biol.* **45**, 771-787.

Ingram, J. and Bartels, D. (1996). The molecular basis of dehydration tolerance in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **47**, 377-403.

Keeling, P. J. and Palmer, J. D. (2008). Horizontal gene transfer in eukaryotic evolution. *Nat. Rev. Genet.* **9**, 605-618.

Kranner, I. and Birtic, S. (2005). A modulating role for antioxidants in desiccation tolerance. *Integr. Comp. Biol.* **45**, 734-740.

Lapinski, J. and Tunnacliffe, A. (2003). Anhydrobiosis without trehalose in bdelloid rotifers. *FEBS Lett.* **553**, 387-390.

Li, X., Romero, P., Rani, M., Dunker, A. K. and Obradovic, Z. (1999). Predicting protein disorder for N-, C-, and internal regions. *Genome Inform. Ser. Workshop Genome Inform.* **10**, 30-40.

Mali, B., Grohme, M. A., Forster, F., Dandekar, T., Schnolzer, M., Reuter, D., Welnicz, W., Schill, R. O. and Frohme, M. (2010). Transcriptome survey of the anhydrobiotic tardigrade Milnesium tardigradum in comparison with *Hypsibius dujardini* and *Richtersius coronifer*. *BMC Genomics* **11**, 168.

Mark Welch, D. and Meselson, M. (2000). Evidence for the evolution of bdelloid rotifers without sexual reproduction or genetic exchange. *Science* **288**, 1211-1215.

Mattimore, V. and Battista, J. R. (1996). Radioresistance of *Deinococcus radiodurans*: functions necessary to survive ionizing radiation are also necessary to survive prolonged desiccation. *J. Bacteriol.* **178**, 633-637.

Potts, M. (1994). Desiccation tolerance of prokaryotes. *Microbiol. Rev.* **58**, 755-805.
Potts, M. (2004). The desiccome: humble beginnings. *Integr. Comp. Biol.* **44**, 624-624.
Pouchkina-Stantcheva, N. N. and Tunnacliffe, A. (2005). Spliced leader RNA-mediated trans-splicing in phylum Rotifera. *Mol. Biol. Evol.* **22**, 1482-1489.
Pouchkina-Stantcheva, N. N., McGee, B. M., Boschetti, C., Tolleter, D., Chakrabortee, S., Popova, A. V., Meersman, F., Macherel, D., Hincha, D. K. and Tunnacliffe, A. (2007). Functional divergence of former alleles in an ancient asexual invertebrate. *Science* **318**, 268-271.
Prilusky, J., Felder, C. E., Zeev-Ben-Mordehai, T., Rydberg, E. H., Man, O., Beckmann, J. S., Silman, I. and Sussman, J. L. (2005). FoldIndex: a simple tool to predict whether a given protein sequence is intrinsically unfolded. *Bioinformatics* **21**, 3435-3438.
Radivojac, P., Obradovic, Z., Brown, C. J. and Dunker, A. K. (2003). Prediction of boundaries between intrinsically ordered and disordered protein regions. *Pac. Symp. Biocomput.* **8**, 216-227.
Ratnakumar, S. and Tunnacliffe, A. (2006). Intracellular trehalose is neither necessary nor sufficient for desiccation tolerance in yeast. *Fems Yeast Res.* **6**, 902-913.
Reardon, W., Chakrabortee, S., Pereira, T. C., Tyson, T., Banton, M. C., Dolan, K. M., Culleton, B. A., Wise, M. J., Burnell, A. M. and Tunnacliffe, A. (2010). Expression profiling and cross-species RNA interference (RNAi) of desiccation-induced transcripts in the anhydrobiotic nematode *Aphelenchus avenae*. *BMC Mol. Biol.* **11**, 6.
Ricci, C. and Covino, C. (2005). Anhydrobiosis of *Adineta ricciae*: costs and benefits. *Hydrobiologia* **546**, 307-314.
Ricci, C., Melone, G., Santo, N. and Caprioli, M. (2003). Morphological response of a bdelloid rotifer to desiccation. *J. Morphol.* **257**, 246-253.
Ricci, C., Caprioli, M. and Santo, N. (2004). Feeding and anhydrobiosis in bdelloid rotifers: a preparatory study for an experiment aboard the International Space Station. *Invertebr. Biol.* **123**, 283-288.
Rice, W. R. and Friberg, U. (2007). Genomic clues to an ancient asexual scandal. *Genome Biol.* **8**, 232.
Rizzo, A. M., Negroni, M., Altiero, T., Montorfano, G., Corsetto, P., Berselli, P., Berra, B., Guidetti, R. and Rebecchi, L. (2010). Antioxidant defences in hydrated and desiccated states of the tardigrade *Paramacrobiotus richtersi*. *Comp. Biochem. Physiol.* **156B**, 115-121.
Schill, R. O., Steinbruck, G. H. and Kohler, H. R. (2004). Stress gene (hsp70) sequences and quantitative expression in *Milnesium tardigradum* (Tardigrada) during active and cryptobiotic stages. *J. Exp. Biol.* **207**, 1607-1613.
Segers, H. and Shiel, R. J. (2005). Tale of a sleeping beauty: a new and easily cultured model organism for experimental studies on bdelloid rotifers. *Hydrobiologia* **546**, 141-145.
Shannon, A. J., Browne, J. A., Boyd, J., Fitzpatrick, D. A. and Burnell, A. M. (2005). The anhydrobiotic potential and molecular phylogenetics of species and strains of *Panagrolaimus* (Nematoda, Panagrolaimidae). *J. Exp. Biol.* **208**, 2433-2445.
Sharon, M. A., Kozarova, A., Clegg, J. S., Vacratsis, P. O. and Warner, A. H. (2009). Characterization of a group 1 late embryogenesis abundant protein in encysted embryos of the brine shrimp Artemia franciscana. *Biochem. Cell Biol.* **87**, 415-430.
Shih, M., Hoekstra, F. and Hsing, Y. (2008). Late embryogenesis abundant proteins. *Adv. Bot. Res.* **48**, 211-255.
Tompa, P. (2009). *Structure and Function of Intrinsically Disordered Proteins*. Boca Raton, FL: Taylor and Francis.
Tompa, P., Szasz, C. and Buday, L. (2005). Structural disorder throws new light on moonlighting. *Trends Biochem. Sci.* **30**, 484-489.
Tunnacliffe, A. and Lapinski, J. (2003). Resurrecting Van Leeuwenhoek's rotifers: a reappraisal of the role of disaccharides in anhydrobiosis. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **358**, 1755-1771.
Tunnacliffe, A. and Wise, M. J. (2007). The continuing conundrum of the LEA proteins. *Naturwissenschaften* **94**, 791-812.
Tunnacliffe, A., Hincha, D. K., Leprince, O. and Macherel, D. (2010). LEA proteins: versatility of form and function. In *Dormancy and Resistance in Harsh Environments* (ed. E. Lubzens, J. Cerda and M. S. Clark), pp. 91-108. Berlin: Springer.
Tyson, T., Reardon, W., Browne, J. A. and Burnell, A. M. (2007). Gene induction by desiccation stress in the entomopathogenic nematode *Steinernema carpocapsae* reveals parallels with drought tolerance mechanisms in plants. *Int. J. Parasitol.* **37**, 763-776.
Watanabe, M., Kikawada, T., Minagawa, N., Yukuhiro, F. and Okuda, T. (2002). Mechanism allowing an insect to survive complete dehydration and extreme temperatures. *J. Exp. Biol.* **205**, 2799-2802.