Anhydrobiosis without trehalose in bdelloid rotifers

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Abstract Eukaryotes able to withstand desiccation enter a state of suspended animation known as anhydrobiosis, which is thought to require accumulation of the non-reducing disaccharides trehalose (animals, fungi) and sucrose (plants), acting as water replacement molecules and vitrifying agents. We now show that clonal populations of bdelloid rotifers *Philodina roseola* and *Adineta vaga* exhibit excellent desiccation tolerance, but that trehalose and other disaccharides are absent from carbohydrate extracts of dried animals. Furthermore, trehalose synthase genes (*tps*) were not found in rotifer genomes. This first observation of animal anhydrobiosis without trehalose challenges our current understanding of the phenomenon and calls for a re-evaluation of existing models.

Key words: Anhydrobiosis; Trehalose; Desiccation tolerance; Bdelloid rotifer

1. Introduction

Anhydrobiosis ('life without water') was first described by van Leeuwenhoek over 300 years ago [1], but the biochemistry on which it is based is still not resolved. Over the last 3 decades, however, considerable emphasis has been placed on a role for the non-reducing disaccharides, chiefly trehalose and sucrose. For example, the anhydrobiotic nematode *Aphelenchus avenae* can produce 10–15% of its dry weight as trehalose when dried slowly, and this is associated with the acquisition of desiccation tolerance [2,3]. The budding yeast *Saccharomyces cerevisiae* also accumulates high concentrations of trehalose during entry into stationary phase and this correlates with increased survival of desiccation [4,5]. Similarly, production of high concentrations of sucrose coincides with maturation and the acquisition of desiccation tolerance in plant seeds [6]; in the resurrection plant *Craterostigma plantagineum*, sucrose represents 90% of total sugar content in dried leaf tissues [7]. These and other reports imply a protective role for disaccharides against extreme water stress, and this is consistent with many in vitro drying experiments where labile proteins and membrane systems are stabilised by sugars [8]. The importance attached to non-reducing disaccharides has led some workers to suggest that not only are such sugars necessary for anhydrobiosis, but that their presence may also be sufficient [9].

However, an increasing amount of evidence, including the following two examples, contradicts this simple hypothesis. Trehalose concentrations during dehydration of anhydrobiotic nematodes, such as *A. avenae*, reach maximal levels before full desiccation tolerance is attained, indicating that additional physiological adaptations are required [3,10,11]. In *Arabidopsis thaliana*, the seeds of certain mutants in the *abi3* locus are unable to withstand desiccation, although sucrose accumulation proceeds normally [12]. Furthermore, if disaccharides are sufficient to achieve anhydrobiosis, it should be possible to confer this ability on a desiccation-sensitive cell by introducing trehalose or sucrose into the cytoplasm, a strategy we have termed anhydrobiotic engineering [13]. However, recent attempts at anhydrobiotic engineering of mammalian cells have met with limited success [13–16]. Such reports imply that disaccharides are not sufficient for anhydrobiosis, and we now present evidence that neither are they necessary in two species of bdelloid rotifer.

2. Materials and methods

2.1. Rotifer culture

Bdelloid rotifers were collected from a bird bath in Cambridge (UK). Rotifer clones P1 (*Philodina roseola*) and A1 (*Adineta vaga*) were established from single individuals, selected by micro-pipette manipulation. Cultures were scaled up for growth in aerated 10 l plastic containers, containing filtered tap water (charcoal filter, Liff Industries, model no. N-SK13, and Nalgene 0.2 μm PES filter). Rotifers were fed *Escherichia coli otsA*$^{-}$ (strain FF2032) [17] which had been grown in Luria–Bertani (LB) medium overnight, recovered by centrifugation and resuspended in distilled water. The bacterial suspensions could be stored at 4°C for up to a week.

2.2. Rotifer harvesting and counting

Rotifers were harvested by briefly increasing the salt concentration of the culture vessel to 0.1 M NaCl, which causes the animals to detach from the tank walls. The medium was subsequently filtered through a Nitex nylon mesh of 20 μm pore size (Precision Textiles), from which rotifers were transferred to tissue culture flasks (Nunc, 1-78883A) after washing several times with distilled water. The total number of rotifers in a flask was estimated by counting animals in squares of known size in five different randomly selected areas of the flask.

2.3. Rotifer and nematode drying

The anhydrobiotic nematode *A. avenae* was grown according to established protocols, described elsewhere [18]. Nematodes were filtered onto a Supor-200 membrane (Pall), exposed to 98% relative humidity (RH) for 3 days and subsequently dried to completion over silica gel. Several methods for drying bdelloid rotifers were used: 'air-dried' rotifers were simply pipetted into an open Petri dish and left to dry to completion at room temperature (approximately 23°C, 33% RH); for other protocols, rotifers were first collected by filtration onto Nitex membrane, after which they were either dried over silica gel ('silica gel-dried'), or exposed to 53% RH for 24 h ('53% RH exposure') in a chamber containing a saturated solution of $Ca(NO_3)_2$ (Merck Index); RH values were checked using a hygrom-

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eter. The most successful method involved placing the nylon membrane carrying the rotifers between two pieces of wet 3MM (Whatman) filter paper, followed by storage in a sealed Petri dish at 25°C in the dark for 2 days, after which the lid was removed and the rotifers air-dried to completeness for an additional 3 days ('preconditioning at 100% RH'). 'Starved' *P. roseola* were not fed for 3 days; 'well-fed' individuals were fed 3 h prior to the experiment. At least 10 batches of 30–50 individuals were dried in different conditions, then re-hydrated, and survival counted 24 h later. Mean survival and standard deviation were calculated accordingly.

2.4. Polymerase chain reaction (PCR)

The following primers were used for amplification of trehalose-6-phosphate synthase (*tps*) genes: 5′-GGACTAGTCAYGAYTAYCAYYTNATG-3′ and 5′-GGAATTCACCAGRTTCATNCCRTC-3′. Primers were derived from regions of the *tps* predicted protein sequence which were highly conserved between the species *Kluyveromyces lactis*, *S. cerevisiae*, *Aspergillus niger*, *Schizosaccharomyces pombe*, *Mycobacterium leprae*, *E. coli* and *Caenorhabditis elegans*. PCR was carried out in 20 μl total volume comprising 75 mM Tris–HCl (pH 8.8 at 25°C), 20 mM $(NH_4)_2SO_4$, 0.01% (v/v) Tween 20, 0.25 mM dNTPs, 1 μM primer DNA (each), 1 μg genomic DNA, 2.5 units Red Hot DNA polymerase (ABgene). PCR was performed in a Hybaid PCR Express thermocycler as follows: 10 cycles of 94°C 45 s, 45°C 45 s and 72°C 1 min; 30 cycles of 95°C 45 s, 60°C 45 s and 72°C 1 min. The reaction products were run on a 1.5% agarose gel and visualised using ethidium bromide. Fragments were excised and recovered using the Hybaid Recovery DNA Purification Kit II; DNA was cloned using a TOPO TA cloning kit (Invitrogen) and subsequently sequenced by the facility at the Department of Genetics, University of Cambridge.

2.5. Residual moisture content

P. roseola were collected on a Nitex membrane and slowly dried with preconditioning at 100% RH according to the protocol given above. The dried rotifers were scraped from the Nitex membrane using a scalpel and transferred to the measuring pan of a thermogravimetric analyser (Perkin-Elmer TGA 7). The initial weight of the sample was determined, and then the analyser was gradually heated up to a final temperature of 150°C which was maintained for 10 min. The weight of the sample was monitored in real time and the water content calculated accordingly; the true value of residual water might be somewhat lower than that determined by this technique, since volatile organics will also be lost on heating.

2.6. Gas chromatography (GC)

Animals were weighed or counted prior to drying (~25 mg *A. avenae*; ~100 000 individuals of *P. roseola* and *A. vaga*) and then transferred into Eppendorf tubes in 1 ml of water. In some experiments, 0.05 mg sucrose was added as an internal standard. Samples were homogenised on ice using a Soniprep 150 ultrasonic disintegrator (Sanyo), ethanol was added to achieve a final concentration of 70%, and the extract was incubated at 70°C for 30 min. Extracts were clarified by centrifugation and concentrated to 1 ml total volume. A chloroform extraction was performed to remove lipids and then the aqueous phase dried at 70°C. Samples were derivatised with 200 μl of a 1:1 mixture of pyridine:*N*,*O*,-*bis*(trimethylsilyl)-trifluoroacetimide (Aldrich Cat. No. 36,057-0 [Poole, UK] and Pierce Cat. No. 38831 [Perbio Science, Tattenhall, UK], respectively) with incubation on a hotplate at 50°C for 45 min. The samples were analysed using a Perkin-Elmer AutoSystem XL gas chromatograph with a DB5 column (Pierce). Control samples analysed included: bacterial food (*E. coli*), rotifer culture medium, Nitex membrane only, water only, trehalose, trehalose-6-phosphate, maltose, sucrose, glucose and other mono-, di- and trisaccharides (data not shown).

Table 1
Desiccation tolerance of *P. roseola*

Protocol	Survival (%)	
	starved *P. roseola*	well-fed *P. roseola*
Air-dried	<1	<1
Silica gel	12±6	18±6
53% RH	29±8	49±8
100% RH	45±9	75±7

Survival (±S.D.) of four different drying protocols of both starved and well-fed animals is shown.

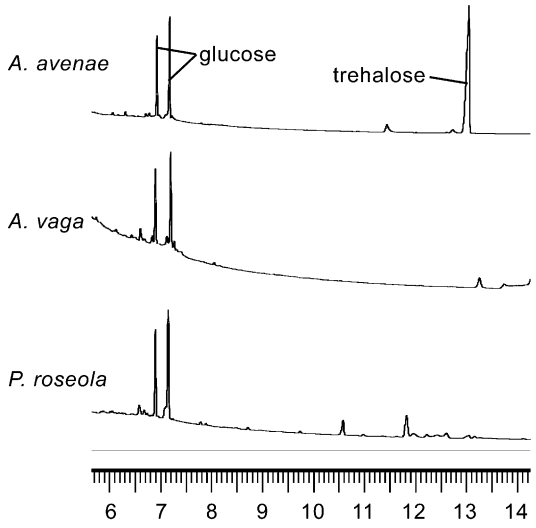

Fig. 1. GC analysis of carbohydrate extracts from nematode and rotifers. A separate experiment is shown for each of three species; the horizontal scale (minutes) shows time of emergence from the GC column: the anomeric forms of glucose emerge as a doublet at ~7 min, whereas trehalose appears at 13 min. In the nematode *A. avenae*, after exposure to 98% RH, trehalose is strongly induced; glucose is also clearly detected. *A. vaga* and *P. roseola* show no trehalose or comparable carbohydrate accumulation on drying, although glucose was present in amounts similar to those in the nematode.

3. Results

3.1. Desiccation tolerance in bdelloid rotifers is protocol-dependent

Clonal cultures of well-fed bdelloid rotifers of the species *P. roseola* and *A. vaga* were subjected to a slow drying regime including preconditioning in a 100% RH environment, after which 75% of the animals was recovered on rehydration (Table 1). Similar results have been reported by Ricci [19]. Thermogravimetric analysis of rotifers dried in this way suggested that a maximum residual moisture content in the range 6–10% of dry weight is achieved, which is similar to that observed for other anhydrobiotic organisms [20].

In contrast, more rapid drying in air or low-humidity environments (i.e. over silica gel or at 53% RH) led to markedly worse survival, suggesting that rotifers, like nematodes, need time to effect certain physiological changes for optimal anhydrobiosis. This view was supported by the fact that starved rotifers showed a reduced ability to undergo anhydrobiosis compared with well-fed individuals, which indicates an energy-expensive process (Table 1).

3.2. Lack of trehalose in bdelloid rotifers

It was expected that at least one of the necessary physiological adaptations in anhydrobiotic rotifers would be induction of trehalose biosynthesis, and therefore carbohydrate extracts of desiccating *P. roseola* and *A. vaga* were prepared for GC. Extracts of the anhydrobiotic nematode *A. avenae* were also prepared as a control, and showed the anticipated high trehalose content. Surprisingly, however, in repeated experi-

ments we were unable to detect any trehalose at all in either species of rotifer tested (Fig. 1). Furthermore, apart from glucose, it was apparent that no other simple sugar was present in significant quantities in dried *P. roseola* and *A. vaga*: mono-, di- and small oligosaccharides would be detected on the chromatograph, as indicated by control experiments using standard reagents. For example, sucrose, when added to the samples as an internal standard prior to extraction, was clearly detected (data not shown).

Glucose comprises ~1% dry weight in the nematode *A. avenae* [21] and similar quantities were present in the rotifers *P. roseola* and *A. vaga*; trehalose constitutes at least 10% dry weight in desiccated nematodes. It is therefore reasonable to assume that we would detect changes in the carbohydrate profile at least in this range (i.e. 1–10% dry weight), which represents the levels of trehalose observed in anhydrobiotic animals [8,9]. In further experiments, trehalose was also not detected in rotifers subjected to salt stress (2 h in 100 mM NaCl; data not shown), which can induce biosynthesis of trehalose or comparable osmolytes in other organisms [22].

3.3. Lack of trehalose synthase genes in rotifer genomes

If rotifers were able to produce trehalose, they would probably use the two-step pathway found in all other eukaryotes. The first step in this pathway is the transfer of glucose from UDPG to glucose-6-phosphate to yield trehalose-6-phosphate, which is catalysed by trehalose-6-phosphate synthase (EC 2.4.1.15), an enzyme whose sequence is highly conserved from bacteria to yeast, nematodes and higher plants [23,24]. A PCR method was devised using degenerate oligonucleotide primers based on conserved protein sequence to clone the respective gene (*tps*) from a wide range of species. Low-stringency conditions (i.e. low initial annealing temperature) were used to allow amplification of fragments which might only have moderate sequence similarity to *tps* genes. However, although *tps* genes were isolated using this technique from species including the budding yeast *S. cerevisiae*, the Gram-negative bacterium *E. coli*, the fruit fly *D. melanogaster* and the nematode *A. avenae*, we have been unable to amplify *tps* PCR fragments from two negative control species (*Mus musculus* and *Homo sapiens*), as well as the two bdelloid rotifer species *P. roseola* and *A. vaga*, indicating that *tps* genes are not present in their genomes (Fig. 2). Two fragments were weakly amplified from *P. roseola* DNA, but more than 50 cloning and sequencing experiments performed on these sequences showed that they did not correspond to *tps* genes, and therefore that they represent false positives.

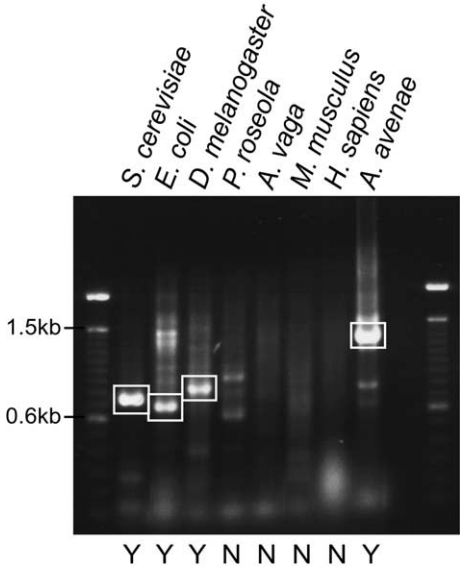

Fig. 2. PCR amplification of trehalose-6-phosphate synthase (*tps*) genes of different species, as indicated above each lane. Y denotes a successful amplification, with the *tps* fragment enclosed in a box; N indicates a negative result. Molecular weight standards (100 bp intervals) are shown in the first and last lanes.

4. Discussion

Although other adaptations associated with anhydrobiosis have been suggested by biochemical and genetic analyses [25,26], the prevalent hypotheses that attempt to explain the phenomenon invoke non-reducing disaccharides, chiefly either trehalose or sucrose, as a major factor [8,9,27]. These sugars are proposed to act as water replacement molecules and as thermodynamic and kinetic stabilisers of biomolecules and membranes. However, the apparent lack of trehalose or an analogue in bdelloid rotifers, which nonetheless exhibit excellent desiccation tolerance, means that these hypotheses, if valid, cannot offer a universal explanation of anhydrobiosis. This point is reinforced by a similar lack of non-reducing disaccharides in other desiccation-tolerant organisms, such as Gram-positive bacteria [28] and some plant seeds [29]. The fact that some anhydrobiotes do not use these sugars does not, of course, mean that they are not important in species where they are found, although we have argued elsewhere that there is a disturbing lack of conclusive evidence for a role of non-reducing disaccharides in living systems [27]. Nevertheless, it seems likely that there are multiple strategies for desiccation tolerance, just as there are for resistance to e.g. salt, or hyperosmotic, stress in different organisms [22,30].

Physiological changes at the protein level might be of more importance than changes in the metabolite pool in bdelloid rotifers undergoing anhydrobiosis. In plants [25,26], and more recently in nematodes [31,32], induction of high levels of hydrophilic proteins – particularly the late embryogenesis abundant (LEA) proteins [33] – has been associated with water stress. LEA-like protein genes have also been described in micro-organisms such as *Haemophilus influenzae* and *Deinococcus radiodurans*, and, in the latter, inactivation of two of these genes results in a significant reduction of desiccation tolerance [34]. Intriguingly, we have recently discovered a dehydration-regulated protein in bdelloid rotifers which is specifically recognised by an antiserum against a nematode LEA protein [35]. Confirmation of this as a rotifer LEA protein will require sequence information, but it seems likely that this fascinating group of proteins is widespread in organisms other than plants, and could have particular relevance for anhydrobiosis. Other adaptations implicated in anhydrobiosis in bdelloid rotifers are expected to emerge from gene discovery and proteomics programmes currently underway. The search for the 'anhydrobiotic gene set', and corresponding 'anhydrobiotic protein set', has been underway for a number of years in plants [25]. A similar strategy in bdelloid rotifers and other animals should lead to a fuller understanding of anhydrobiosis in these organisms.

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References

[1] Van Leeuwenhoek, A. (1702) Letter to Hendrik van Bleyswijk, dated 9th February, in: (van Rijnberk, G. and Palm, L.C., Eds.), The Collected Letters of Antoni van Leeuwenhoek, Vol. 14, pp. 55–73, Swets and Zeitlinger, Amsterdam, 1999.
[2] Madin, K.A.C. and Crowe, J.H. (1975) J. Exp. Zool. 193, 335–342.
[3] Perry, R.N. (1999) Parasitology 119, S19–S30.
[4] Gadd, G.M., Chalmers, K. and Reed, R.H. (1987) FEMS Microbiol. Lett. 48, 249–254.
[5] Hottiger, T., Boller, T. and Wiemken, A. (1987) FEBS Lett. 220, 113–115.
[6] Oliver, M.J., Tuba, Z. and Mishler, B.D. (2000) Plant Ecol. 15, 85–100.
[7] Bianchi, G., Gamba, A., Murelli, C., Salamini, F. and Bartels, D. (1991) Plant J. 1, 355–359.
[8] Crowe, J.H., Carpenter, J.F. and Crowe, L.M. (1998) Annu. Rev. Physiol. 60, 73–103.
[9] Crowe, J.H. and Crowe, L.M. (1992) in: Water and Life (Somero, G.N., Osmond, C.B. and Bolis, C.L., Eds.), pp. 87–103, Springer, Berlin.
[10] Higa, L.M. and Womersley, C.Z. (1993) J. Exp. Zool. 267, 120–129.
[11] Womersley, C.Z. and Higa, L.M. (1998) Nematologica 44, 269–291.
[12] Ooms, J.J.J., Léon-Kloosterziel, K.M., Bartels, D., Koornneef, M. and Karssen, C.M. (1993) Plant Physiol. 102, 1185–1191.
[13] García de Castro, A., Lapinski, J. and Tunnacliffe, A. (2000) Nat. Biotech. 18, 473.
[14] Guo, N., Puhlev, I., Brown, D.R., Mansbridge, J. and Levine, F. (2000) Nat. Biotechnol. 18, 168–171.
[15] García de Castro, A. and Tunnacliffe, A. (2000) FEBS Lett. 487, 199–202.
[16] Chen, T., Acker, J.P., Eroglu, A., Cheley, S., Bayley, H., Fowler, A. and Toner, M.L. (2001) Cryobiology 43, 168–181.
[17] Kaasen, I., Falkenberg, P., Styrvold, O.B. and Strøm, A.R. (1992) J. Bacteriol. 174, 889–898.
[18] Evans, A.A.F. (1969) Nematology 2, 99–100.
[19] Ricci, C. (1998) Hydrobiologia 387/388, 321–326.
[20] Potts, M. (1994) Microbiol. Rev. 58, 755–805.
[21] Browne, J.A. (2001) Ph.D. Thesis, National University of Ireland, Maynooth.
[22] Strøm, A.R. (1998) J. Biosci. 23, 437–445.
[23] Vogel, G., Aeschbacher, R.A., Müller, J., Boller, T. and Wiemken, A. (1998) Plant J. 13, 673–683.
[24] Blázquez, M.A., Santos, E., Flores, C.L., Martinez-Zapater, J.M., Salinas, J. and Gancedo, C. (1998) Plant J. 13, 685–689.
[25] Bartels, D. and Salamini, F. (2001) Plant Physiol. 127, 1346–1353.
[26] Hoekstra, F.A., Golovina, E.A. and Buitink, J. (2001) Trends Plant Sci. 6, 431–438.
[27] Tunnacliffe, A. and Lapinski, J. (2003) Philos. Trans. R. Soc. Lond. B 358, 1755–1771.
[28] Linders, L.J.M., Wolkers, W.F., Hoekstra, F.A. and van 't Riet, K. (1997) Cryobiology 35, 31–40.
[29] Bewley, J.D. and Black, M. (1994) Seeds: Physiology of Development and Germination, Plenum Press, New York.
[30] Zhu, J.K. (2002) Annu. Rev. Plant Biol. 53, 247–273.
[31] Browne, J., Tunnacliffe, A. and Burnell, A. (2002) Nature 416, 38–38.
[32] Solomon, A., Salomon, R., Paperna, I. and Glazer, I. (2000) Parasitology 121, 409–416.
[33] Cuming, A.C. (1999) in: Seed Proteins (Shewry, P.R. and Casey, R., Eds.), pp. 753–780, Kluwer Academic, Dordrecht.
[34] Battista, J.R., Park, M.-J. and McLemore, A.E. (2001) Cryobiology 43, 133–139.
[35] Tunnacliffe, A., Lapinski, J. and McGee, B. (2004) Hydrobiologia, in press.