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Trehalose in desiccated rotifers: a comparison between a bdelloid and a monogonont species

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Abstract

In response to drought bdelloid and monogonont rotifers undergo anhydrobiosis and are assumed to synthesize protective chemicals, which are commonly sugars. In contrast to most anhydrobionts, bdelloids have earlier been shown to lack trehalose as protective chemical, and more importantly to lack trehalose synthase (tps) genes. It remains to be assessed if the absence of trehalose is a characteristic common to the entire taxon Rotifera, or if it is limited to bdelloids, or is peculiar to the two bdelloid species investigated so far. In this study, anhydrobiotic adults of a bdelloid species (*Macrotrachela quadricornifera*) and resting eggs of a monogonont species (*Brachionus plicatilis*) were analysed by thin layer chromatography and gas chromatography to detect the presence of trehalose. No trehalose was detected in the bdelloid, while the anhydrobiotic resting egg of the monogonont rotifer contained about 0.35% trehalose of its dry weight. Although very little, the presence of trehalose in *B. plicatilis* suggests that the trehalose synthase genes, absent in bdelloid rotifers, are present in non-bdelloid rotifers.

Keywords: Anhydrobiosis; Resting eggs; Trehalose; Bdelloid rotifer; Monogonont rotifer; Protective chemicals; Osmolyte

1. Introduction

Cryptobiosis is a widespread strategy characterized by a reversible arrest of development and metabolism. Cryptobiosis is common to several organisms in response to adverse conditions of the habitat, like drought, cold, osmotic stress, etc.; when cryptobiosis is induced by drought it is termed anhydrobiosis (Keilin, 1959; Crowe, 1971). Anhydrobiosis may be tolerated by any life stage, that is by the egg, embryo, juvenile and adult (i.e. Tardigrada, Rotifera Bdelloidea, Nematoda) (Crowe and Madin, 1975; Wright et al., 1992; Ricci, 1998), or by one ontogenetic stage, only (i.e. embryos of Crustacea, Rotifera Monogononta, larvae of certain Insecta) (Hinton, 1960; Clegg, 1978; Pourriot and Snell, 1983).

To survive anhydrobiosis, organisms synthesise various "protective" substances. The most common ones are non-reducing disaccharides, either trehalose in microbes, animals, and lower plants, or sucrose in higher plants (Crowe et al., 1992; Clegg, 2001). Sugars can be effective alone or in a mixture and play a central role in stabilising membranes. Not only disaccharides have been found to protect lipids, but also monosaccharides, like glucose, if combined with hydroxyethyl starch (e.g. Crowe et al., 1997).

Trehalose, in particular, seems to be present in almost every animal capable of surviving anhydrobiosis and has been proposed to function as a "water replacement" molecule and stabilize the structure of macromolecules and membranes during desiccation (Webb, 1965; Crowe et al., 1998). The amount of trehalose in anhydrobiotic animals

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can be high, such as 18% dry weight (DW) in cysts of *Artemia franciscana* (Clegg, 1965), and in the chironomid larva *Polypedilum vanderplanki* (Watanabe et al., 2002), but also as low as 0.2% DW in the nematode *Steinernema carpocapsae* (Womersley, 1990) or 2.3% DW in the tardigrade *Adorybiotus coronifer* (Westh and Ramløv, 1991). The variation of the amount of trehalose appears irrespective of the resting stage (e.g. both embryos of *A. franciscana* or adults of *Aphelenchus avenae* have similar trehalose percentages), of the taxon (e.g. in the nematodes trehalose varies between 0.2% and 12% DW) (Behm, 1997) and of the habitat (predictable or unpredictable, cyclical or temporal) (Càceres, 1997).

Recently, two species of bdelloid rotifers, *Philodina roseola* and *Adineta vaga*, were found to lack trehalose when anhydrobiotic and, more relevant, to lack trehalose synthase (tps) genes (Lapinski and Tunnacliffe, 2003; Tunnacliffe and Lapinski, 2003). This finding demonstrates that trehalose may be absent in animals capable of anhydrobiosis and questions the role of this sugar as "the" molecule for animals that protect their tissues against the damages due to desiccation. However, it remains to be assessed if the absence of trehalose is a feature of the two bdelloid species only or if it is a trait of all bdelloids or is a trait common to all rotifers. If the lack of trehalose is limited to the species investigated so far, the sugar should be found in other Bdelloid species. If the trait is common to other bdelloids, we can presume that all bdelloids do not 'need' that molecule to undergo anhydrobiosis. Alternatively, absence of trehalose could be characteristic of the whole taxon Rotifera. In either case, other possible mechanisms should be investigated, since almost all bdelloids are able to survive desiccation and several monogononts produce resting eggs, which are capable of desiccation (Gilbert, 1974; Ricci, 1998; Schroeder, in press).

Among the Rotifera, both monogononts and bdelloids are frequently exposed to desiccation in their natural habitats, and are desiccation tolerant, but each rotifer group follows a different strategy (Ricci, 2001). Monogononts live in cyclical habitats, and possess one resting stage only, called a resting egg, which is an arrested embryo. To produce this, the monogononts detect species-specific factors and initiate a complex cascade of reproductive events: monogonont females shift from female-producing ameiotic parthenogenesis (thelytoky) to male-producing meiotic parthenogenesis (arrhenotoky), and finally, mating with the haploid male (mixis), produce the resting egg. For a given time, the arrested embryo does not respond to any stimulus, and resumes development after a series of environmental and internal stimuli, that are often species-specific and are not necessarily linked to harsh environmental conditions (Gilbert, 1977, 2003; Pourriot and Snell, 1983). Bdelloids live in unpredictable temporal habitats; their dormant stage may consist of the egg as well as of the adult rotifer, and dormancy is broken as soon as the conditions that initiated it are removed (Ricci, 1998; Ricci et al., 1987).

The two bdelloid species found to lack trehalose , *P. roseola* and *A. vaga*, are both able to survive drought by anhydrobiosis and belong to two different orders, Philodinida and Adinetida (Melone and Ricci, 1995). The former is a typical 'aquatic' species and the latter is very common in almost any habitat. Whether the absence of trehalose and of related genes is a peculiarity of the two species or is due to their habitat is to be ascertained. Alternatively, the absence of trehalose, shared by *P. roseola* and *A. vaga*, might be a trait of all bdelloids. Bdelloid and Monogonont rotifers have several morphological and molecular similarities (i.e., Wallace et al., 1996; García Varela et al., 2000; Mark Welch, 2000), and are expected to use similar molecules as protective sugars in their dormant stages. In this study, anhydrobiotic adults of an additional bdelloid species, *Macrotrachela quadricornifera*, and the resting eggs of the monogonont *Brachionus plicatilis* were analysed by thin layer chromatography (TLC) and gas chromatography (GC) to assess the presence of trehalose, checking morphological integrity of both dormant stages and recording their viability.

2. Materials and methods

B. plicatilis Müller, 1786 is a brackish water species to which crowding induces the mictic phase and the production of resting eggs after a series of parthenogenetic generations (Gilbert, 2003). Our strain of *B. plicatilis* is called CCB1, and has been cultivated under laboratory conditions (12‰ medium salinity) during several generations. The resting egg production was induced experimentally by promoting mictic phase, and the eggs were collected from the culture bottom, transferred to a filter paper and desiccated at room temperature at about 60% relative humidity. Replicate samples of approx. 250 resting eggs were prepared to be processed for chemical analysis. About 7 days after desiccation, one sample with about 70 resting eggs was rehydrated by adding water medium (12‰salinity); hatching was recorded 24–48 h after rehydration to record egg viability.

M. quadricornifera Milne, 1886 is a freshwater species; the strain used in this study has been cultivated in our laboratory for several years. As with all bdelloids, this species can be made anhydrobiotic by removing the water medium. Bdelloids were transferred from the culture to filter paper, and desiccated in a humido-thermostatic chamber for 76 h (for details, see Ricci et al., 2003). Each sample of anhydrobiotic bdelloid rotifers had 600 reproductive adults. After 7 days of desiccation, one sample with about 50 anhydrobiotic *M. quadricornifera* was rehydrated by adding culture medium, and recovery rate was recorded 24 h after hydration.

For each species, in addition to the sample used to assess viability, six dry samples were prepared. Of these samples,

one was processed for SEM analysis, two samples were analysed using thin layer chromatography, three samples were analysed with gas chromatography.

Additional samples containing dry *B. plicatilis* resting eggs and anhydrobiotic *M. quadricornifera* were prepared to measure the dry weight, used in the calculation of the amount of trehalose per egg. Six replicate samples of 20–30 dry resting eggs each and four replicate samples of 40 anhydrobiotic bdelloids were prepared and weighted by a Mettler Toledo AT automatic electro-balance (Table 1). Each sample weight was referred to either single egg or animal, respectively, and the figures were averaged among replicates.

2.1. Scanning electron microscopy

Dry resting eggs of *B. plicatilis* and anhydrobiotic specimens of *M. quadricornifera* were fixed with OsO_4 vapour for 2 h (Ricci et al., 2003). All samples were mounted on stubs, sputter-coated with gold and observed under a LEO 1430 scanning electron microscope.

2.2. Thin layer chromatography (TLC)

Two samples of dry *B. plicatilis* resting eggs and two samples of anhydrobiotic *M. quadricornifera* were analysed by Silica-gel thin layer chromatography (TLC) to determine the presence of sugars and polyols. Each sample was dissolved in 300 μL 100% ethanol, warmed to 90 °C for 10 min, bath-sonicated for 15 min, and centrifuged at 5000×*g* for 3 min. The supernatant was collected. This extraction procedure was repeated twice. The supernatants were pooled and frozen at −80 °C for 1 h and freeze dried for 24 h. The resultant pellet was re-suspended in 10 μL 50% ethanol, and vortex-mixed. Five microliters of the suspension was loaded on a thin layer chromatography alufoil plate (Silica Gel 60 F_{254}, Merck). Sugars and polyols used as standards were: Glucose, Fructose, Sucrose, Rhamnose, and Trehalose. The mixture was obtained by mixing: Dulcitol, Myo-inositol, Mannitol, Trehalose, and Glucose, in equal parts.

Sugars and polyols were dissolved in 50% ethanol. Standard concentrations were 1 mg/mL for single carbohydrates and 5 mg/mL for the mixture. Five microliters of the carbohydrates and 1 μL of the mixture were loaded on the TLC plate.

Table 1
Dry weight (μg) of resting eggs of *B. plicatilis* and anhydrobiotic adults of *M. quadricornifera* (mean±S.E.)

Species	Stage	Replicates (#)	Single weight (μg)
B. plicatilis	resting egg	7 (175)	0.31±0.03
M. quadricornifera	adult	4 (160)	1.72±0.14

The samples were prepared groupings of eggs or adults, and the weight was calculated for each single egg or adult.

The eluent used for the separation of the carbohydrates was a solution of ethylacetate/acetic acid/methanol/water (60:15:15:10). A solution of 5% hydrogen sulphate, 5% acetic acid, 0.5% anisaldehyde in deionised water was used to stain the sugars and heated at 120 °C for 10 min.

2.3. Gas chromatography (GC)

Three samples of 250 dry monogonont resting eggs and three samples of 600 anhydrobiotic bdelloids were processed. Each sample was transferred to centrifuge tubes containing 200 μL 40% ethanol, heated to 100 °C for 5 min, sonicated four times for 2 min each on ice in a Vibra Cell (Sonics and Materials Danbury, USA) and centrifuged at 6000×*g* for 12 min. The supernatant was collected and transferred to a 300-μL GC-vial to be processed. The pellet was extracted three times in 150 μL 96% ethanol at 100 °C for 3 min, sonicated on ice four times for 2 min each, and centrifuged at 6000×*g* for 12 min. The supernatants obtained after the three extractions were pooled into GC-vials, and dried under a stream of nitrogen at 65 °C. The pellet was re-suspended in 20% ethanol, heated at 100 °C for 3 min, sonicated on ice four times for 2 min each, and centrifuged at 6000×*g* for 12 min. The supernatant obtained was transferred to GC-vials, and dried under a stream of nitrogen at 65 °C for 90 min. To each GC vial was added 100 μL 0.1 M sorbitol as internal standard, and the sample dried under a stream of nitrogen for 60 min to absolute dryness. The samples were converted to their trimethylsilyl derivatives by adding 70 μL Sigma-Sil-A, vortex-mixed and dried under a stream of nitrogen at 65 °C for 90 min. To the vials were added 20 μL of Sigma-Sil-A and they were capped and vortex-mixed.

For the detection of the peaks in the GC chromatograms as well as for the quantification of the sugars found, the following substances: Dulcitol, D-Sorbitol, Mannitol, D-Glycerol, myo-Inositol, D-Fructose, D-Glucose, α-L-Rhamnose, Sucrose and D-Trehalose (Sigma) were used as standards and derivatized as described above.

Standards and samples were injected into an OV 1701 column on a Hewlett Packard 5890 series II gas chromatograph equipped with a FID detector. Nitrogen was used as carrier gas, the injection port was held at 300 °C and the detector temperature was 300 °C. The temperature program in the oven was: 100 °C for 2 min, a gradient from 100 to 250 °C in 10 min and finally held at 250 °C for 7 min. Qualitative identification of trehalose was obtained by comparing chromatograms of samples and standards and quantification was based on the internal sorbitol standard (this polyalcohol could not be detected in crude extracts).

The method followed in this study is similar to that described by Westh and Ramløv (1991).

3. Results

Resting eggs of *B. plicatilis* were nicely oval and their shell showed a fairly rough surface (Fig. 1A). Desiccated specimens of *M. quadricornifera* were contracted into a tun shape, and their body extremities were fully withdrawn, transversal grooves on the dorsal part and longitudinal folds on either side were visible (Fig. 1B). One resting egg of *B. plicatilis* weighed 0.31 (±0.03, S.E.) μg, and an anhydrobiotic *M. quadricornifera* weighed 1.7 (±0.14, S.E.) μg (Table 1). The recovery rates were recorded 24 h after rehydration; 80% *B. plicatilis* dry resting eggs hatched, and 88% dry *M. quadricornifera* resumed activity. Thus, the samples processed for the detection of sugars consisted of anhydrobiotic stages of both taxa.

The presence of sugars and polyols in monogonont resting eggs and in bdelloid adults was investigated by Silica-gel TLC. This approach revealed the presence or absence of a given chemical, but was not used to quantify the amount. In the lane with *M. quadricornifera* adults, no evident spots were detected, except a faint spot in correspondence to glucose (Fig. 2). In contrast, the *B. plicatilis* egg lane presented one spot with the same retention time as the trehalose standard, and this was clearly visible (Fig. 2). Using this analysis, no other sugars were detected in the *B. plicatilis* lane.

By GC analysis, the amount of trehalose in the monogonont resting eggs was determined on three replicate samples.

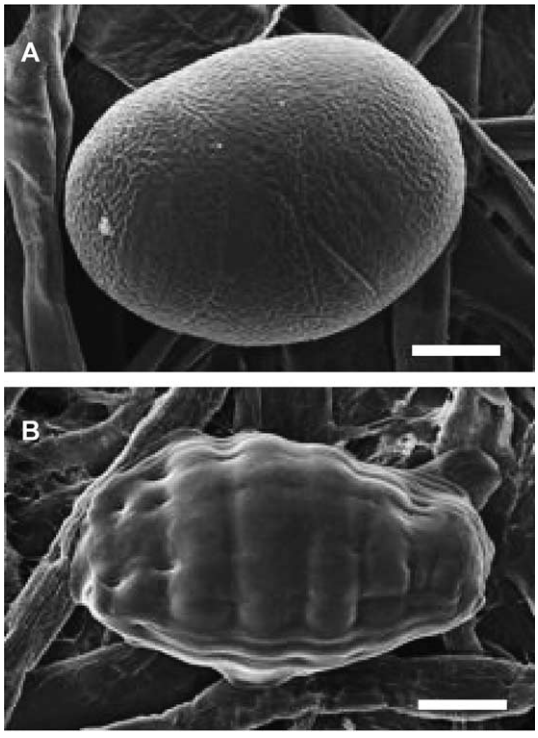

Fig. 1. Scanning electron microscopy images of anhydrobiotic stages of rotifers. (A) *B. plicatilis* resting egg. Bar, 25 μm. (B) *M. quadricornifera* adult. Bar, 25 μm.

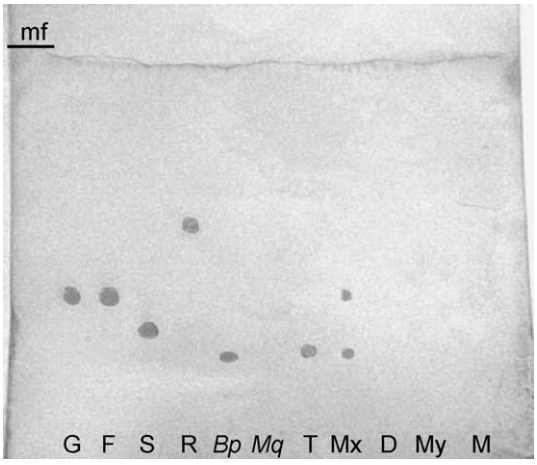

Fig. 2. Analysis by thin layer chromatography (TLC) of sugars and polyols in resting eggs of *B. plicatilis* (*Bp*) and anhydrobiotic adults of *M. quadricornifera* (*Mq*). Trehalose was detected in the resting eggs of *Bp* but not in *Mq*. Reference sugars were glucose (G), fructose (F), sucrose (S), rhamnose (R), trehalose (T), mixture (Mx), dulcitol (D), myo-inositol (My), mannitol (M). Migration front, mf.

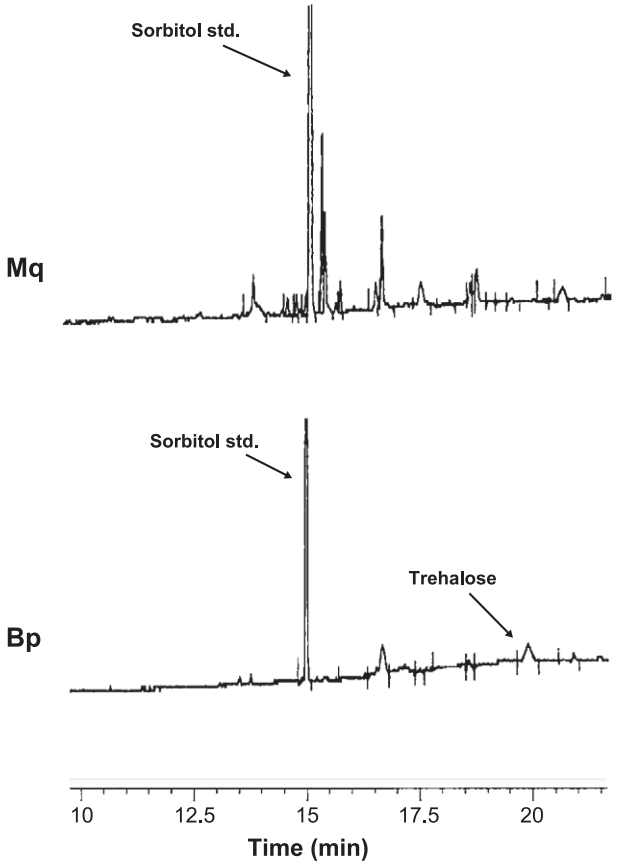

Fig. 3. Analysis by gas chromatography (GC) of sugars and polyols in resting eggs of *B. plicatilis* (*Bp*) and in anhydrobiotic adults of *M. quadricornifera* (*Mq*). Trehalose was detected in the resting eggs of *Bp* but not in *Mq*. In both chromatograms, the peak corresponding to Sorbitol, the internal standard, is indicated. The abscissa refers to retention time, in minutes (min).

Known amounts of sorbitol were added to standards and to samples to have an internal standard, and the amount of trehalose was calculated on the basis of the sorbitol peak. It was then referred to the mass of a single dry egg. The resting egg contained 1.09 ($\pm$0.4, S.E.)$\times 10^{-3}$ μg trehalose, that corresponded to 3.52×10^{-3} μg trehalose/μg DW, or 0.35% (Fig. 3).

It should be noted that in *M. quadricornifera* trehalose was not detected, neither with TLC nor with GC. Glucose was detected in *M. quadricornifera* with both TLC and GC but only in very small amounts which could not be quantified to get a reliable figure.

4. Discussion

The lack of detectable trehalose in *M. quadricornifera* corroborates the results obtained by Lapinski and Tunnacliffe (2003) on the two bdelloids, *P. roseola* and *A. vaga*. *M. quadricornifera* and *P. roseola* belong to one clade (order Philodinida), and the other one, *A. vaga*, belongs to a different clade (order Adinetida) (Melone et al., 1998). If the absence of trehalose is ascribed to the absence of tps (trehalose synthase) genes in *A. vaga* and *P. roseola* (Lapinski and Tunnacliffe, 2003; Tunnacliffe and Lapinski, 2003), it seems likely that the same genes are also lacking in *M. quadricornifera*, supporting the hypothesis that absence of these genes might be a condition common to the entire taxon Bdelloidea. If this is true, all bdelloids do not synthesize trehalose as a protection of their biological structures during desiccation because they do not posses the biochemical tools for producing it.

In contrast, the resting egg of the monogonont *B. plicatilis* contains trehalose, although its amount is very small. Surprisingly, the amount of trehalose in the *B. plicatilis* resting egg is less than that reported in most known anhydrobiotic stages. For instance, 'cysts' of *A. franciscana* contain about 18% trehalose of dry weight (Clegg and Conte, 1980). But on the other hand, the amount of trehalose found in the resting egg is not dissimilar from that of some nematodes, like the third larval stage (L_3) of *S. carpocapsae* or L_2 of *Anguina tritici* (Behm, 1997). Both cysts of *A. franciscana* and resting eggs of *B. plicatilis* live in similar habitats and consist of arrested embryos that can survive desiccation. Both taxa are presumably adapted to cope with salt stress as active adults as well as resting stages. The role of trehalose in *B. plicatilis* resting eggs might then be that of an organic osmolyte rather than a desiccation protective chemical. Considering the size of the resting egg and the amount of trehalose measured and assuming a water content in the resting egg of ca. 60% (on the basis of preliminary observation, M.C. and the size of the resting eggs), the trehalose concentration in the fully hydrated egg can be calculated to amount to approximately 100 mM, assuming that no trehalose is broken down or lost during rehydration. This figure is within the range at which compatible osmolytes are found in other organisms, especially if several substances form the complement of organic osmolytes (Hochachka and Somero, 1984). If such hypothesis is correct, we should expect that adult *B. plicatilis* possess trehalose as well, but the present study did not address this point.

Whether in large or small amount, the presence of trehalose implies that the tps genes are present in a monogonont rotifer (*B. plicatilis*), thus other non-bdelloid rotifers might be expected to have the genes. Since trehalose apparently is present in almost every anhydrobiotic animal, it is more parsimonious to suppose that bdelloids have lost the metabolic pathway leading to this sugar, and the lack of trehalose may be synapomorphic to bdelloids only.

At present, the synthesis of alternative sugars, like sucrose, as protective chemicals during desiccation seems not to be the case for the bdelloids, while Late Embryogenesis Abundant (LEA) proteins have been hypothesised to be involved in the protection of the structures during anhydrobiosis (Browne et al., 2002; McGee et al., in press). Nevertheless, other chemicals might play a similar role (Crowe et al., 1997). Present results on a bdelloid rotifer (*M. quadricornifera*) coupled to previous evidence on two other bdelloid species (*P. roseola* and *A. vaga*) prompt to reconsider trehalose as "the" chemical universally used by the animals and associated with the acquisition of desiccation tolerance.

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