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# Molecular snapshot of an intracellular freezing event in an Antarctic nematode

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ABSTRACT

The Antarctic nematode, *Panagrolaimus* sp. DAW1 (formerly called *Panagrolaimus davidi*), is the best documented example of an organism able to survive intracellular ice formation in all of its compartments. Not only is it able to survive such extreme physiological disruption, but it is able to produce progeny once thawed from such a state. In addition, under slower rates, or less extreme degrees, of cooling, its body remains unfrozen and the vapour pressure difference between the supercooled body fluids and the surrounding ice leads to a process termed cryoprotective dehydration. In contrast to a fairly large body of work in building up our molecular understanding of cryoprotective dehydration, no comparable work has been undertaken on intracellular freezing. This paper describes an experiment subjecting cultures of *Panagrolaimus* sp. DAW1 to a range of temperatures including a rapid descent to −10 °C, in a medium just prior to, and after, freezing. Through deep sequencing of RNA libraries we have gained a snapshot of which genes are highly abundant when *P.* sp. DAW1 is undergoing an intracellular freezing event. The onset of freezing correlated with a high production of genes involved in cuticle formation and subsequently, after 24 h in a frozen state, protease production. In addition to the mapping of RNA sequencing, we have focused on a select set of genes arising both from the expression profiles, as well as implicated from other cold tolerance studies, to undertake qPCR. Among the most abundantly represented transcripts in the RNA mapping is the zinc-metalloenzyme, neprilysin, which also shows a particularly strong upregulated signal through qPCR once the nematodes have frozen.


## 1. Introduction

Apart from the work of Salt in describing intracellular freezing in the large fat body cells and labial glands of the goldenrod gall fly, *Eurosta solidaginis*, in the late 1950's [44–46,50], the study of the phenomenon of intracellular freezing *in vivo* has remained little understood, and little explored. However, some recent physiological studies on the Antarctic nematode, *P.* sp. DAW1 [38–40,67–70,72,73], provides the necessary background to now bring molecular techniques to bear on an extraordinary adaptation.

A free-living bacteriovorous nematode, *Panagrolaimus* sp. DAW1 [61] (previously called *Panagrolaimus davidi*, see Raymond & Wharton [40]), was first isolated from the McMurdo Sound region of the Antarctic in 1989 and cultured at the University of Otago [66]. The culture was found to survive intracellular freezing [68], with the ability to produce progeny afterwards. To date, *P.* sp. DAW1 remains the best documented case of such survival, even among other Antarctic nematodes [30,51,74]. *P.* sp. DAW1 also has the ability to undergo cryoprotective dehydration when subjected to high sub-zero temperatures or slower rates of cooling [71,73]. However, while an increasingly detailed molecular picture is emerging of cryoprotective dehydration [8,9,18,57–59], no comparable molecular work has been undertaken on the survival of intracellular freezing at the whole organism level. The study of *P.* sp. DAW1 provides an increasingly detailed physiological understanding of intracellular ice formation on which to build up a molecular picture of the process.

While there are many organisms able to withstand ice formation in extracellular spaces [1,10,16,17,20,26,53,54,64,65], the ability to withstand such disruption within the cells, and survive with the ability to produce progeny afterwards, is much rarer. In *P.* sp. DAW1, although the cuticle is a sufficient barrier to ice, at low sub-zero

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temperatures inoculative freezing can spread through the excretory pore and other orifices, with the ice seeding the body fluid and freezing all, including intracellular, compartments [68,73]. One of the ideas on the more lethal consequences of this freezing is the physical damage to the cell membranes once the smaller ice crystals that have initially formed, themselves begin to cluster together, or recrystallise [23,38,73]. Traditionally the term antifreeze protein has been used to designate any protein involved in the inhibition of freezing. Recently, some authors have argued for a more general term for proteins that play a role in ice formation and in combating ice recrystallisation [12,72]. These ice-active proteins (IAP) can then be classed according to one of three types: Ice nucleating proteins (INP), which help to initiate ice formation; antifreeze proteins (AFP), which prevent ice nucleation by creating a thermal hysteresis (TH) between the melting and freezing point of a sample in the presence of an ice crystal; and recrystallisation-inhibition proteins (RIP), which inhibits recrystallisation, the aforementioned process in which larger ice crystals form at the expense of smaller more numerous crystals. Although most RIPs also have at least low levels of thermal hysteresis activity, in some cases extracts of organisms with RIP activity have to be concentrated to a high degree before TH activity can be detected. RIPs tend to be found in freeze tolerant species while AFPs (with more substantive TH) are mostly present in freeze avoiding species. However, in *Panagrolaimus* sp. DAW1, given the extreme freezing disruption that it can experience, and the fact that it is freeze tolerant, it is expected that its IAPs will not include AFPs [38], but rather RIPs. A further clue on the possible importance of RIPs in the survival of intracellular freezing, comes from a recent study by Raymond and Wharton [40], where the smaller the sizes of the ice crystals is correlated positively with the survival rate. But to date, no IAP of any kind has been isolated in *P.* sp. DAW1 [60], even though they have been shown to play a role in freezing tolerance [72].

Among other important classes of genes are those involved in the sugar biosynthetic pathways (such as those producing trehalose), Late Embryogenesis Abundant (LEA) proteins, chaperone proteins (such as the heat shock proteins), and enzymes involved in antioxidant metabolism. These all play a role in the process of cryoprotective dehydration, and have been detected in *P.* sp. DAW1 [60]. While it is not clear what role, if any, these play in the nematodes' survival of intracellular freezing, it would be highly surprising if they were not involved.

The extent of survival of *P.* sp. DAW1 when subjected to intracellular freezing is dependent, however, on at least two properties. Wharton and To [69] have shown decreased survival ability when under either hyper- or hypo-osmotic stress. While Raymond and Wharton [39,40] have shown that nutritional status is an important factor, possibly related to the fact that glycogen levels decrease when under nourished. These two studies imply that unless in a relatively stress-free state, *Panagrolaimus* sp. DAW1 is unable to endure the extreme physiological disruption of intracellular freezing, suggesting that the physiological conditions that allow for a sudden rate of cooling and intracellular freezing are at the very limit of its physiological endurance.

With all the physiological work that has been undertaken a number of key insights have been gained, yet there remains little understanding at the molecular level. To address this, we have generated sets of transcripts expressed in *P.* sp. DAW1 when the nematodes have been cooled rapidly to a relatively low sub-zero temperature (−10 °C), both before and after (immediately and post 24 h) the medium has frozen. These treatments were compared with controls that include stages in which *P.* sp. DAW1 is expected to undergo both cold acclimation and cryoprotective dehydration. This approach provides a snapshot of highly abundant transcripts from genes novel to the intracellular freezing process and distinct from those generally involved in responses to stress. We have further validated the results through qPCR analysis on genes selected both from within the transcript analysis, as well as those implicated in other cold tolerance studies.

## 2. Materials and methods

### 2.1. Culturing and experimental design

The culturing and experimental treatments of the nematodes consisted of control (culturing temperature and cold acclimation), cryoprotective dehydration and intracellular freezing stages. The control and cryoprotective stages consisted of the following:

*Treatment 1,* culture temperature*: P.* sp. DAW1 were cultured in S medium at 20 °C for 3 weeks and fed every 3–4 days with *Escherichia coli* [39].

*Treatment 2,* cold acclimation*:* After treatment 1, the nematodes were exposed to cold acclimation by keeping them at +5 °C for 3 days. This duration at +5 °C was used because longer periods of acclimation can be detrimental to the ability to survive intracellular freezing, due to the effects of starvation. This acclimation regime is known to produce about 80% survival in nematodes frozen at −10 °C, at which temperature all individuals freeze intracellularly [39,40].

Nematodes from treatments 1 and 2 were extracted for the subsequent experiments using a modified Baermann technique [19] and transferred to 1.5 ml microcentrifuge tubes in a balanced salt solution (BSS) [36] before snap freezing in liquid nitrogen and storage at −70 °C.

*Treatment 3,* cryoprotective dehydration*:* After cold acclimation, nematodes were extracted and transferred to 1 ml of BSS in 1.5 ml microcentrifuge tubes and placed in an aluminium holder immersed in the bath of a refrigerated circulator. The sample was cooled from +1 °C to −1 °C at 0.5 °C $min^{-1}$, and the sample frozen by adding a small ice crystal and maintained at −1 °C for 24 h. Under these conditions, the medium freezes but not the nematodes.

*Treatment 4,* cryoprotective dehydration thawing*:* After treatment 3 the nematodes were warmed to +1 °C at 0.5 °C $min^{-1}$ and allowed to recover for 24 h at 20 °C.

The intracellular freezing stages (the stages in which ice is formed inside the cells of P. sp DAW1) consisted of the following:

*Treatment 5U*, −10 °C in unfrozen media: The samples were cooled to −10 °C at 0.5 °C $min^{-1}$. When this temperature was reached, it was noted whether the medium had frozen or not, and those in unfrozen medium removed for extraction.

*Treatment 5F*, −10 °C in frozen media: The samples were cooled to −10 °C at 0.5 °C $min^{-1}$ as with Treatment 5U. When this temperature was reached samples that had frozen spontaneously during cooling were removed and labelled.

*Treatment 6*, −10 °C for 24 h: Samples subjected to treatment 5F were kept frozen at −10 °C for 24 h.

*Treatment 7*, thawing and recovery from intracellular freezing: Samples subjected to treatment 6 were warmed to +1 °C at 0.5 °C $min^{-1}$ and then kept at 5 °C for 24 h. Subsamples (four 2 μl samples diluted 4 × with BSS and incubated at 5 °C) were taken to determine survival and the proportion moving, after a physical stimulus (expelling from a pipette), counted at intervals up to 24 h, after which no additional increase in survival rate would be expected. The time taken for 50% of the nematodes to recover, and 95% confidence limits (CL), was determined using probit analysis in SPSS after $\log_{10}$ transformation of time [31].

After treatment, nematodes were snap frozen in liquid nitrogen before storage at −70 °C, ready for RNA extraction.