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The *Caenorhabditis elegans parvulin* gene subfamily and their expression under cold or heat stress along with the *fkb* subfamily

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ABSTRACT

Parvulins and FKBPs are members of the peptidyl–prolyl *cis*/*trans* isomerases (PPIase) enzyme family whose role is to catalyze the interconversion between the *cis trans* forms of a peptide bond preceding internal proline residues in a polypeptide substrate. Members of the *parvulin* subfamily have been found to be involved in a variety of diseases, including Alzheimer's disease and cancer and are also considered possible antiparasitic targets. Genes *Y110A2AL.13* (*pin-1*) and *Y48C3A.16* (*pin-4*) were found in the worm's genome, possibly encoding *parvulins*. One is homologous to human and fly PIN1 whereas the other is homologous to human and fly PIN4. Both were expressed in *Escherichia coli*, purified and found to have in vitro PPIase activity. Expression levels of both genes, as well as the *fkb* genes (that encode FK506-binding proteins) were measured during development and under cold or heat stress conditions. The results revealed a potential role for these genes under temperature-related stress. RNAi silencing was performed for wild type and mutant strain worms under normal and cold or heat stress conditions. A reduced lifespan was observed when *pin-4* dsRNA was fed to the *fkb-5* deficient worms. Our work presents a first attempt to characterize the *Caenorhabditis elegans parvulins* and may present an interesting starting point for further experimentation concerning their role, along with the FKBP subfamily, in nematode physiology and their possible use as antiparasitic targets.

1. Introduction

Parvulins, along with cyclophilins (cyclosporin A-binding proteins) and FKBPs (FK506-binding proteins) are the three known subfamilies of the peptidyl-prolyl cis/trans isomerase enzyme family (PPIase, EC 5.2.1.8). These enzymes are ubiquitously distributed in many cellular compartments and a significant number of them have been identified in eukaryotes, prokaryotes and archaea [1,2]. Their function is to catalyze the interconversion between the *cis trans* forms of a peptide bond preceding internal proline residues in a polypeptide substrate, thus playing a significant role in protein folding and transport, RNA splicing and the regulation of multi-protein complexes in cells [1,3,4]. It has been found that the mis-folding of proteins may lead to polymorphisms with variable properties which can lead to toxic protein accumulation, aggregation and loss of function. Such cases have been described as having a potential role in malignancy [4].

The binding of cyclophilins and FKBPs with immunosuppressants, cyclosporin A and rapamycin respectively (and other compounds) that have strong inhibitory effects on certain parasites in culture and in animal models of infection, as well as their presence in various parasitic protozoa and helminths makes them possible antiparasitic targets [5]. *Caenorhabditis elegans* is an ideal model for such research and so far almost all cyclophilins and FKBPs in the worm's genome have been studied [2,6–8].

Cyclophilins are the largest subfamily of PPIases having 17 members identified in humans and *C. elegans*, 14 in *Drosophila melanogaster* and 8 in *Saccharomyces cerevisiae* [1]. They all carry a domain that binds to the widely used immunosuppressive agent cyclosporin A.

FKBPs are less conserved than cyclophilins [9]. So far, 13 FKBPs have been identified in the human genome, 8 in *C. elegans*, 8 in *D. melanogaster* and 4 in *S. cerevisiae* [1]. In *C. elegans* double and triple FKBP mutants were found to exhibit a cold-sensitive larval lethal phenotype. This defect also revealed a role in molting, cuticle collagen expression, hypodermal seam cell morphology and the structural integrity of the cuticular extracellular matrix [7]. *C. elegans* FKB-6 contains a tetratricopeptide repeat motif (TPR), similar to the human orthologues FKBP51 and FKBP52. An interaction between FKB-6 and the C-terminal DAF-21 pentapeptide MEEVD was observed using NMR. This is consistent with interactions found between the human immunophilin TPR domains and human Hsp90 [2].

Most organisms studied so far have a small number of *parvulins* (2 in humans and *C. elegans*, 3 in *D. melanogaster* and 1 in *S. cerevisiae*). They seem to have one *parvulin* homologous to human PIN1 and another homologous to human PIN4 (in higher eukaryotes) [1].

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Pin-1 is the most studied *parvulin* gene with evidence that it is involved in a variety of diseases, including Alzheimer's disease and cancer where it is overexpressed in many cell types [10]. *Pin-1* has recently been shown to be overexpressed in mouse skin cells exposed to UVA radiation and is also associated with UVA-induced AP-1 activation [11].

2. Materials and methods

2.1. Gene sequences, strains and vectors

Wild-type *C. elegans* strain N2 (Bristol) and deletion mutant strains *fkb-2(ok3007)I* RB2222, *fkb-5(ok653)I* VC467 and *fkb-4(ok240)V* RB1213, were obtained from the Caenorhabditis Genetics Center (CGC). Strains *daf-16(mu86)* CF1038 and *daf-2(el370)* CB1370 were kindly provided by Dr. P. Syntichaki (BRFAA, Athens).

Genes encoding putative *parvulins* were identified by BLAST queries within http://wormbase.org/. Clones yk1571f12 and yk1407d10 that contain EST's corresponding to gene *Y48C3A.16* were requested from Y. Kohara's lab (NIG, Japan). The full coding sequence was then amplified by PCR. As there were no clones available with EST's corresponding to gene *Y110A2AL.13*, the full coding sequence was amplified by PCR from *C. elegans* cDNA. The primers, restriction enzymes and vectors used are available upon request. All sequences were verified by sequencing. Conserved domains were identified using CDD BLAST (http://ncbi.nlm.nih.gov/). Localization of proteins was predicted using PSORT II (http://psort.org/).

2.2. Isolation of total RNA and cDNA

Total RNA was prepared by homogenization of mixed-stage populations of worms that were grown at 20 °C on NGM plates with an OP50 lawn. This was done using a monophasic solution of phenol and guanidine isothiocyanate (TRIzol™ Reagent, Gibco-BRL) according to the manufacturer's instructions. For the analysis of temporal expression patterns, synchronized populations of worms were grown in liquid cultures (S medium containing concentrated OP50) and total RNA was isolated from eggs, L1, L2 and L3 stage larvae (mixed), L4 stage larvae, adult worms and dauers [12].

For the analysis of expression patterns under stress conditions, mixed-stage populations of worms were grown on NGM plates with OP50 at 20 °C. The plates were later moved to 10 °C for the cold stress or 30 °C for the heat stress. Total RNA was isolated from worms collected 2 and 24 h after moving the plates to the stress conditions, in order to follow the response of the expression levels before experiencing problems with lethality. Plates grown at 20 °C were used for the control.

Isolated total RNA was treated with DNase to remove genomic DNA contamination followed by phenol/chloroform extraction. Poly(A)$^+$-RNA was reverse transcribed to cDNA using SuperScript II RNase H-Reverse Transcriptase and Oligo (dT)12–18 Primer (Invitrogen), according to the manufacturer's instructions.

2.3. Quantitative real-time RT–PCR

Primers for real-time RT-PCR analysis were designed close to the 3′ end of the spliced gene sequences using Primer3 (http://primer3.sourceforge.net/). Real-time RT-PCR was performed with an Applied Biosystems 7500 PCR system. For the analysis of temporal expression patterns, relative mRNA levels of the gene of interest (X) were calculated as a ratio to the mean of two ubiquitously expressed genes *act-1* [13] and *ubq-2* [14] (A) as $(1+E)^{-\Delta C_t}$, where ΔC_t was calculated as $C_t^X C_t^A$, [15]. The efficiency of the reactions (E) was calculated using LinRegPCR software (http://gene-quantification.de) [16]. All measurements were taken in triplicate using cDNA from three repetitions of the experiments.

2.4. Recombinant expression in Escherichia coli and enzyme activity assay

Competent BL-21(DH3) *E. coli* cells were transformed with a pET-28a vector for the expression of *Y48C3A.16* and *Y110A2AL.13*. Six histidines (His-Tag) were added to the amino-terminus of the two proteins. Expression and purification was performed as described before [15]. PPIase activity of recombinant *C. elegans parvulins* was measured as previously described [17,18]. The specificity constants k_{cat}/K_M were determined using the equation $k_{cat}/K_M = (k_b - k_u)/[E]$, where k_b is the first-order rate constant of the PPIase catalyzed reaction, k_u is the constant of the PPIase uncatalyzed reaction, and $[E]$ is the PPIase concentration in the assay, assuming that the entire amount of pure enzyme at total concentration represents catalytically active molecules. The stability of recombinant *parvulins* in the presence of a-chymotrypsin was tested by pre-incubation with the amount of a-chymotrypsin used in the assay reaction (0.45 mg/ml) prior to the addition of the peptide substrate and the measurement for PPIase activity. All measurements were performed in triplicate.

2.5. RNAi experiments

RNAi was conducted by feeding using HT115 *E. coli* transformed with the pL4440 vector containing the sequences of interest. Synchronized L3 stage worms were obtained by NaOH/chlorine bleaching [12]. RNAi plates were made by seeding 50 μl of bacterial strains (grown overnight in LB) onto NGM plates containing 50 μg/ml ampicillin (Amp) and 1 mM IPTG. Plates were left overnight at RT to induce expression of dsRNA. Worms were transferred to RNAi plates and were kept at 20 °C [19]. Worms were monitored for various phenotypes. *E. coli* strains transformed with an empty pL4440 vector as well as a strain expressing *gpb-1* dsRNA (that produces an embryonic lethal phenotype) were used as controls. RNAi by feeding was also conducted on plates grown initially at 20 °C and then moved to 10 °C for cold stress or 30 °C for heat stress.

For the lifespan assay, 25 synchronized L3 worms (progeny from worms already fed with the bacteria expressing dsRNA) per construct were monitored every 24 h and transferred to fresh plates every 3–4 days. Worms were scored as dead when they failed to move when touched with a platinum wire. Worms fed *E. coli* HT115 transformed with an empty pL4440 vector were used as a control and the experiment was repeated twice. All RNAi experiments were preformed with WT *C. elegans* strain N2 as well as the *fkb-2*, *fkb-4*, *fkb-5*, *daf-16* and *daf-2* mutant strains. Statistical analysis for the lifespan assays was performed using GraphPad Prism 4 software.

3. Results

3.1. In silico analysis

Two genes were identified in the worm's genome that encode putative *parvulins*. These are *Y110A2AL.13* and *Y48C3A.16*. The first gene is located on chromosome II and has a 486 bp coding sequence consisting of five exons. The product of this gene has 161 amino acids and a molecular weight of 18.3. It is homologous to human PIN1, *D. melanogaster* DODO and *S. cerevisiae* PIN1 with a similarity of 98.1%. For this reason, gene *Y110A2AL.13* and its product will be referred to as *pin-1* and PIN1 respectively. The sec-

ond gene is also located on chromosome II and has a coding sequence of 381 bp consisting of two exons. The product has 126 amino acids and a molecular weight of 13.3. It is homologous to human PIN4 and fly CG11858-PA with a similarity of 99.2%. This gene and its product will be referred to as *pin-4* and PIN4 respectively. A gene named *pin-2* in wormbase refers to a member of the PINch protein family and is not related to PPIases. The two worm proteins share a homology of 24.2% and while PIN1 carries a PPIase and a WW domain (dystrophin), PIN4 carries only a PPIase domain. Though no signal peptide was identified in these proteins, they were both predicted as nuclear using PSORT II.

3.2. Quantitative real-time RT-PCR

The measurement of transcript accumulation showed that the two putative *parvulin* genes are expressed at relatively low levels throughout development (Fig. 1). The maximum expression levels, relative to *act-1* and *ubq-2*, was found during the egg stage and reached 0.06 and 0.15 for *pin-1* and *pin-4* respectively. The *fkb* genes revealed different expression patterns. The highest levels were seen for *fkb-1* and *fkb-2* that were approximately two times those of the reference genes at the adult and egg stage respectively. The lowest levels were observed for *fkb-4* and *fkb-8* that were similar to those of *pin-1* and *pin-4*. Genes *fkb-3*, *fkb-5*, *fkb-6* and *fkb-7* were expressed at high levels at the egg stage and *fkb-3* and *fkb-6* were also expressed at high levels from the L4 stage onward. During the larval stages L1, L2, and L3 transcript accumulation was extremely low for all genes except *fkb-2*.

The expression levels of the *pin* and *fkb* genes were also measured after mixed-stage populations of worms were left for 2 or 24 h at 10 °C for cold stress or 30 °C for heat stress. For the cold stress, the results showed an increased expression of *pin-1* and *pin-4* (Fig. 2A). This was greater for *pin-4*, where the relative expression reached approximately 0.6 after 24 h at 10 °C. For *fkb-2* and *fkb-5* there was a reduced expression, compared to the control and for the rest of the *fkb* genes, no significant change was observed. For the heat stress, again there was an increased expression of *pin-1* and *pin-4* (Fig. 2B). However, the increase was greater than under cold stress and was similar for both genes, reaching a relative expression of 0.8, after 24 h at 30 °C. Regarding the *fkb* genes, it was *fkb-1* that showed a great increase in relative expression, reaching 1.6, while *fkb-2* and *fkb-5* were expressed at much lower levels, compared to the control treatment. A smaller increase of expression was also observed for *fkb-3*, *fkb-6* and *fkb-7*, while *fkb-4* and *fkb-8*, showed no change.

3.3. Expression in E. coli and enzyme activity assay

Expression in *E. coli* BL-21(DH3) cells transformed with pET-28a containing inserts of *pin-1* and *pin-4* produced recombinant proteins containing a His-tag at the amino-terminus. Solubility was satisfactory for both proteins. Purity and integrity of enzymes eluted from the NiNTA columns was confirmed by SDS–PAGE analysis. The concentration of the enzyme solutions after purification ranged from 0.2 to 4 mg/ml (determined by Bradford technique).

The results from the photometric assay for the purified recombinant PINs showed PPIase activity for both when using Leu in the place of Xaa in the substrate. For PIN1 we measured $k_{cat}/K_M = 1.7 \pm 0.4 \times 10^6\ M^{-1}\ s^{-1}$ and for PIN4 we measured $k_{cat}/K_M = 4.4 \pm 0.9 \times 10^4\ M^{-1}\ s^{-1}$. Enzymatic activity was too low or not reproducible when using Ala, Glu, or Arg.

3.4. RNAi experiments

No visible phenotype was observed after feeding worms with bacteria expressing *pin-1* or *pin-4* dsRNA (at 20 °C). The results were the same for plates grown, initially at 20 °C, and then moved to 10 °C for cold stress or 30 °C for heat stress. These experiments were also performed with the *fkb-2*, *fkb-4*, *fkb-5*, *daf-16* and *daf-2* mutant strains, again without producing a visible phenotype. The control treatment with *gpb-1* dsRNA produced a phenotype of 100% embryonic lethality.

The lifespan assay performed (at 20 °C) with the WT and the mutant strains revealed a statistically significant reduction in the worm's lifespan by 36% (from 14 days mean to 9 days) when *pin-4* dsRNA was fed to the *fkb-5* mutant strain (Fig. 3). A shorter, yet still significant, reduction in the worm's lifespan by 11% was also observed for the *fkb-2* deletion strain, when silencing *pin-4*. No statistically significant change in the worm's lifespan was observed

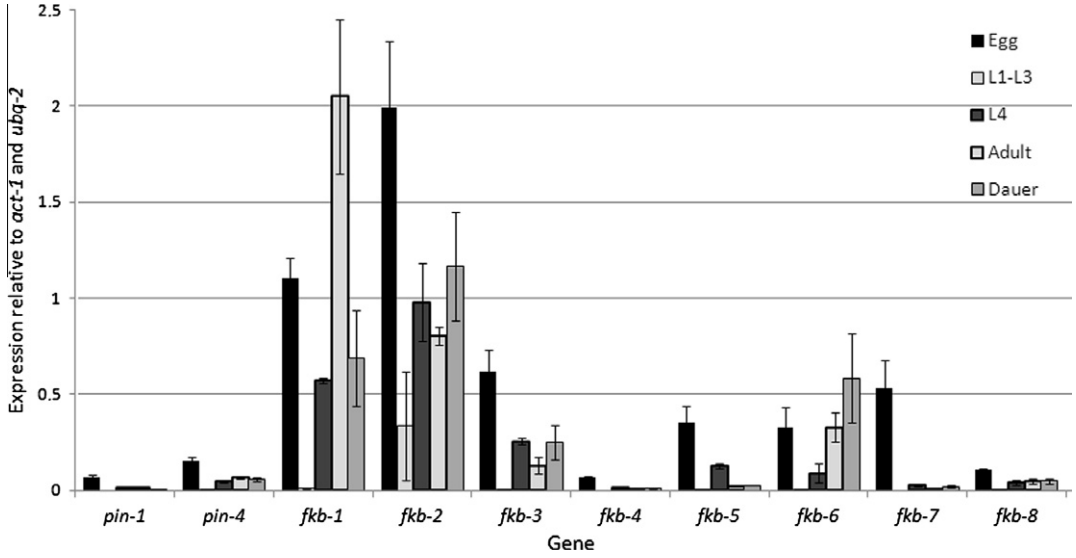

Fig. 1. Accumulation of *pin* and *fkb* transcripts during different developmental stages in *C. elegans*. Total RNA was isolated from synchronized worm cultures (at 20 °C). The measurement of transcript accumulation showed that the two putative *parvulin* genes are expressed at relatively low levels compared to the *fkb* genes, throughout development. The maximum expression levels were found during the egg stage and reached 0.06 and 0.15 for *pin-1* and *pin-4* respectively (relative to *act-1* and *ubq-2*). The bars show means with SE.

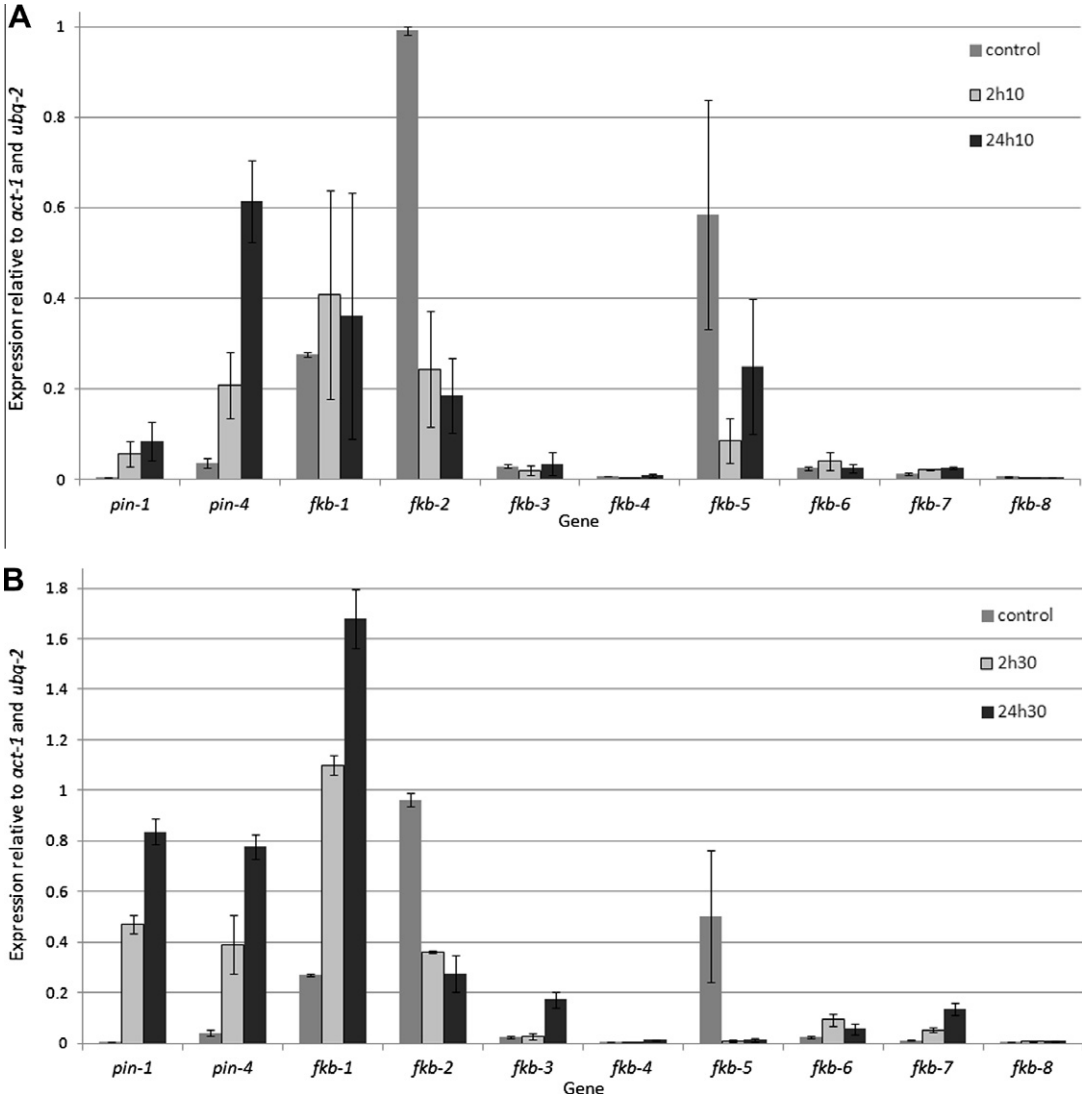

Fig. 2. Accumulation of *pin* and *fkb* transcripts in *C. elegans* after 2 and 24 h at (A) 10 °C and at (B) 30 °C. Total RNA was isolated from mixed-stage worm cultures after 2 or 24 h at 10 °C for cold stress or at 30 °C for heat stress. The measurement of transcript accumulation showed an increased expression of *pin-1* and *pin-4* in both cases. Cultures kept at 20 °C were used for the control. The bars show means (relative to *act-1* and *ubq-2*) with SE.

for the rest of the treatments (data not shown for *daf-2* and *daf-16* strains).

4. Discussion

The worm's genome contains two genes encoding *parvulin*-type PPIases. The gene *pin-1* (*Y110A2AL.13*) encodes an enzyme almost identical to human, fly and yeast PIN1. This is made obvious by amino acid alignment (not shown). The same applies for gene *pin-4* (*Y48C3A.16*) that encodes a *parvulin* almost identical to human and fly PIN4. A phylogenetic tree describing these relationships has already been published [1].

Our experiments showed that both PIN1 and PIN4 have in vitro PPIase activity. With $k_{cat}/K_M = 1.7 \pm 0.4 \times 10^6\ M^{-1}\ s^{-1}$ and $k_{cat}/K_M = 4.4 \pm 0.9 \times 10^4\ M^{-1}\ s^{-1}$, for PIN1 and PIN4 respectively, their activity falls within the range of the previously characterized *C. elegans* FKB3 ($k_{cat}/K_M = 0.189 \pm 0.039 \times 10^6\ M^{-1}\ s^{-1}$), [7] and bovine FKB ($k_{cat}/K_M = 0.66 \pm 0.12 \times 10^6\ M^{-1}\ s^{-1}$) [18], but significantly higher than those of *parvulins* such as murine PIN1 ($k_{cat}/K_M = 6.21 \times 10^3\ M^{-1}\ s^{-1}$), [20] and human PAR14 ($k_{cat}/K_M = 1012 \times 10^3\ M^{-1}\ s^{-1}$), [21]. These results can be expected, given the aforementioned similarity of these enzymes to other PPIases. The PSORT II prediction was nuclear, for both, which also conforms with data presented in the bibliography [1].

Previous studies have shown that PIN1 is expressed in the following compartments: pharynx, intestine, rectal gland cells, rectal epithelium, hypodermis, seam cells, excretory cell, nervous system, nerve ring, ventral nerve cord, head neurons and tail neurons [22,23]. For PIN4, expression was described as being predominantly in intestinal cells, and in other cells in the head and tail (presumably neurons), and the pharynx and hypodermis of young larvae [24].

The interconversion between the cis trans forms of a peptide bond, catalyzed by PPIases, is strongly dependent on temperature [18]. At low temperatures, one would assume that the need for PPIases to increase the rate of such reactions would be grater. At higher temperatures, however, the possible expression of heat-shock proteins may make speculation more difficult. It has been shown that double (*fkb-4* and *fkb-5*) and triple (*fkb-3*, *fkb-4* and *fkb-5*) deletion mutants arrest at 12 °C [7]. *C. elegans* is normally grown at temperatures between 16 °C and 25 °C [12]. We measured the transcript accumulation for the two *pin* and the eight *fkb* genes throughout development and under mild, non lethal, cold or heat stress conditions. While transcript accumulation does not

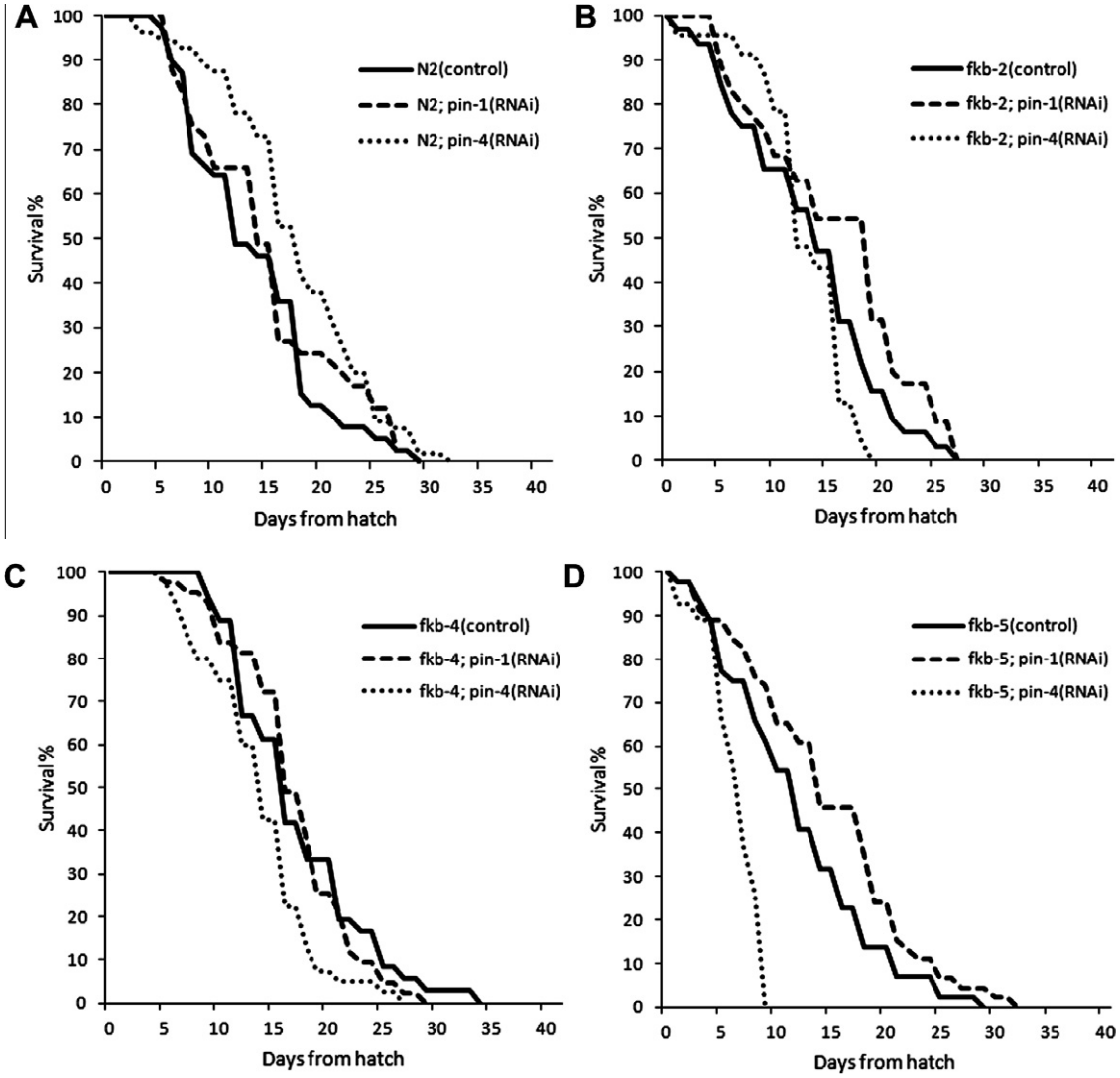

Fig. 3. Lifespan of *C. elegans* strains N2 (A), *fkb-2* (B), *fkb-4* (C) and *fkb-5* (D) fed bacteria expressing *pin-1* or *pin-4* dsRNA (at 20 °C). The results revealed a reduction in the worm's lifespan when *pin-4* dsRNA was fed to the *fkb-5* mutant strain. A shorter reduction was also observed for the *fkb-2* deletion strain, when silencing *pin-4*. No change in the worm's lifespan was observed for the rest of the treatments. An empty pL4440 vector was used for the controls.

necessarily describe the concentration and/or the activity of the actual proteins, as seen in Fig. 1, the two *parvulin* genes are expressed at quite low levels. However, when the worms are exposed to a low temperature (10 °C), their expression levels increase to levels comparable to those of *fkb-1*, *fkb-2* and *fkb-5* (Fig. 2A). Under these conditions, the increased expression of *pin-4* indicates a possible role in the worm's adaptation, when most of the *fkb* genes show a reduced expression. This appears to be the case for both *pin* genes when the worms are exposed to the heat stress (30 °C) (Fig. 2B). Here, both genes, as well as *fkb-1*, appear to be part of a physiological reaction to the stress. Overall, both the *pin* genes and the *fkb* genes seem to react differently to the varying temperatures. This is interesting since *pin-1* has already been shown to play a role in saving the cell cycle from G0 arrest and *pin-1*$^{-/-}$ mice showed phenotypes similar to cyclin D1-deficient mice, suggesting that *pin-1* is involved in the G1/S progression by regulating cyclin D1 function [25]. Cyclin D1 synthesis, on the other hand, has been shown to be induced by mild heat shock in cell cultures [26]. We therefore decided to screen for possible phenotypes after silencing *pin-1* and *pin-4* using wild-type worms as well as three available deletion mutant strains for *fkb* genes. As expression of *fkb-3*, *fkb-4* and *fkb-5* has been found to be influenced by the DAF-2 insulin-like pathway, [27] we also included the *daf-2* and *daf-16* mutant strains. The RNAi experiments were performed at a normal (22 °C), low (10 °C) or high (30 °C) temperature where the role of these genes was suspected to be more significant (according to the real-time RT-PCR results). The only phenotype scored, however, was the reduced lifespan of the *fkb-5* deletion strain fed with *pin-4* dsRNA by 36% (Fig. 3). This shows that, although these two genes (*pin-2* and *fkb-5*) may not be expressed at very high levels, under normal conditions, at least one of them seems to be required for a normal lifespan. A shorter reduction (11%) in the worm's lifespan was also observed for the *fkb-2* deletion strain, when silencing *pin-4*. Even if *parvulins* are indeed involved in the cell cycle through cyclin D1 regulation, we can assume that their importance may only become obvious in *C. elegans* under particular conditions such as stress or lack of other PPIases.

Finally, we conclude that the worm's genome contains two genes encoding *parvulin*-type PPIases. Both PIN1 and PIN4 are active enzymes and are expressed at relatively low levels throughout development. The role of these genes may be more important for the worm's physiology than is apparent at first sight, particularly under low or high temperatures. Their expression levels are strongly influenced by temperature related stress conditions and

together with members of the *fkb* gene family, the expression of *pin-4* may be crucial for the worm's lifespan.

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