Loss of Secretory Pathway FK506-Binding Proteins Results in Cold-Sensitive Lethality in *Caenorhabditis elegans.*

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Abstract.

The FK506-binding proteins (FKBs) represent ubiquitous enzymes that catalyse the rate-limiting peptidyl prolyl *cis-trans* isomerization step in protein folding. The nematode *Caenorhabditis elegans* has eight FKBs, three of which (FKB-3, -4 and -5) have dual peptidyl prolyl *cis-trans* isomerase (PPIase) domains, signal peptides and ER-retention signals. PPIase activity has been detected for recombinant FKB-3. Both FKB-3 and -5 are expressed in the exoskeleton-synthesising hypodermis with transcript peaks that correspond to the molting and collagen synthesis cycles. FKB-4 is expressed at a low level throughout development. No phenotypes were observed in deletion mutants in each of the secretory pathway FKBs. Combined triple and *fkb-4/-5* double deletion mutants were found to arrest at 12°C, but developed normally at 15-25°C. This cold-sensitive larval lethal effect was not maternally-derived, occurred during embryogenesis and could be rescued following the transgenic introduction of a wild type copy of either *fkb-4* or *fkb-5*. The temperature-sensitive defects also affected molting, cuticle collagen expression, hypodermal seam cell morphology and the structural integrity of the cuticular extracellular matrix. This study establishes that the secretory pathway FK506-binding PPIase enzymes are essential for normal nematode development, collagen biogenesis and the formation of an intact exoskeleton under adverse physiological conditions.

Introduction

The FK506 binding proteins (FKBPs or FKBs) belong to a group of proteins that have peptidyl prolyl *cis-trans* isomerase (PPIase; EC 5.2.1.8) activity, and together with the cyclophilins (CYPs or CYNs) are collectively called the immunophilins. FKBs and CYPs are structurally unrelated and have high affinities for the structurally distinct immunosuppressive drugs FK506 and cyclosporin A, respectively (Bell *et al.*, 2006). In addition, there is no link between PPIase activity and the immunosuppressive action of these compounds. The immunophilins have a widespread distribution in nature being found in bacteria, plants and man. However, the endogenous physiological functions are poorly understood but include possible roles in protein translation, folding, assembly and trafficking (Pemberton and Kay, 2005; Bell *et al.*, 2006). These enzymes stabilise the *cis-trans* transition state, accelerate the isomerization event and therefore promote protein folding and assembly of multi-protein complexes.

The collective roles of the FKBs and CYPs have been addressed following the generation of single and multiple mutants in the budding yeast *Sacchromyces cerevisiae*. No overt phenotypes were observed, indicating that they are dispensable for normal development in this simple unicellular organism (Dolinski *et al.*, 1997). The examination of immunophilin function in multicellular organisms has likewise been relatively uninformative raising the possibility of redundancy of function and the possible non-essential nature of these genes. In relation to PPIase activity, this is supported by the fact that proline isomeration will proceed in the absence of a PPIase catalyst, albeit at a slow rate, particularly at lower temperatures (Kofron *et al.*, 1991; Wang and Heitman, 2005). The major exception to the lack of associated phenotype for an immunophilin mutant has been described for the *ninaA* cyclophilin gene of *Drosophilia*. This gene product is involved in rhodopsin folding and mutations result in protein misfolding leading to blindness (Schneuwly *et al.*, 1989; Stamnes *et al.*, 1991).

Cis-trans isomerization of peptidyl prolyl bonds has been established as being a slow rate-limiting step in the folding of numerous proteins, in the particular proline-rich collagens (Bächinger, 1987; Steinmann *et al.*, 1991) and tropoelastin (Patterson *et al.*, 2000). Nematode worms are encased in a collagen-rich exoskeleton called the cuticle that is synthesized in its entirety five times during normal development. The model organism *Caenorhabditis elegans* has been proposed as an excellent model system to dissect extracellular matrix (ECM) formation (Kramer, 1997) and to study the enzymes and chaperones involved in its biogenesis and deposition (Page and Winter, 2003).

Collagens represent essential structural proteins in all multicellular animals and are characterized by the repeat motif Gly-X-Y, where X and Y are commonly proline and hydroxyproline respectively. The cuticle collagens represent a large multigene family that encodes small proline-rich collagens with interrupted triple helical regions. The collagens are synthesized in waves of expression that correspond to the molting cycle, a process that permits growth and defines the separate larval and adult forms and their distinct cuticles. The completely sequenced *C. elegans* genome encodes eight separate FKB genes, five of which are involved in the secretory pathway (Bell *et al.*, 2006). Of these isoforms, FKB-3, -4 and -5 have dual PPIase domains, a signal peptide and an ER-retention signal sequence. Here we examine the individual and collective functional significance of these FKBs in *Caenorhabditis elegans*. The collective phenotypes together with the shared temporal and spatial expression patterns confirms that an essential role is played by these enzymes in the folding of the nematode collagens and the biogenesis of the ECM.

Materials and Methods

Deletion mutants- The *fkb-3(tm348)* homozygous viable deletion allele was isolated by Dr. Shohei Mitani, National Bioresource Project for the Nematode, using UV/TMP as a mutagen. A 1487 base pair deletion in C05C8.3 (*fkb-3*) on chromosome V, from -1128 to +360 relative to ATG start of *fkb-3* gene was created, that removed the promoter region plus all of the first exon of *fkb-3.* This is predicted to be a null mutant. The strain TP8 was made by outcrossing five times with wild type N2 nematodes using the following primers: tm348F1, 5' gacgaatgatgatcgaagttag 3'; tm348F2, 5' tcatggaatggaaattgcaatg 3'; tm348R1, 5' tcatcagtgatccacttggctc 3'. The *fkb-4(ka4)* homozygous viable deletion mutant in ZC455.10 on chromosome V was isolated using the mutagen UV/TMP following published protocols (Barstead, 1999) (deletion screen primers are available on request). The deletion region was cloned and sequenced revealing a 527bp deletion in *fkb-4* extending from +247 to +772 relative to ATG, removing the majority of exons 2 and 3. The strain TP65 was generated following backcrossing four times to N2 using the following primers; Fkb-4BCF1, cgaaccacttgtttcctg 3'; Fkb-4BCF2, 5' gatcagattcacaagattgaag 3'; Fkb-4BCR1, 5' catttacctatatgcggttgg 3'. An additional homozygous viable deletion mutant, named *fkb- 4(ok240),* was obtained from the international *C. elegans* Gene Knockout Consortium. The deletion site was cloned and sequenced using primers ILS 5' tcgaagaaaagacgagcacc 3' and ok240F1 5' caggaatcacagcgtcgata 3' and found to extend from -1478 to +48 relative to the ATG start of *fkb-4*. The strain RB1213 was then backcrossed 4x with N2 using

the following primers; ok240F1; ok240R1, 5' catagcctctctatccattccag 3'; ok240R2, 5' gagcctctatactatcagac 3'. A *fkb-5(tm475)* homozygous viable deletion allele isolated and obtained from Dr. Shohei Mitani. The allele *tm475* is a 446 bp deletion on chromosome I covering C50F2.6 (*fkb-5*) removing 237 bp of the *fkb-5* sequence, starting in the last intron and completely removing all 180 bp of the exon 4 and 209 bp of downstream sequence. *tm475* is a predicted to be a null allele due to the removal of the C-terminal ER-retention motif from the FKB-5 protein. The strain TP9 was made by backcrossing four times with wild type N2, using the following primers; tm475F1, 5' ggatgttatagacgttctaac 3'; tm475R1, 5' ttgcatctggaagtgtcg 3'; tm475R2, 5' gatccgtctagtgat 3'. All additional nematodes strains used in these studies were obtained from the *C. elegans* Genetics Centre.

Maintenance and manipulation of nematodes- Nematode strains were maintained following standard culture methods (Brenner, 1974). L1 cultures were synchronised following bleach treatment of gravid hermaphrodites and single worm PCR was performed as described previously (Barstead, 1999). The combined mutant strains TP73 [*fkb-4(ka4)*V*; fkb-5(tm475)*I], TP81 [*fkb-3(tm348) fkb-4(ok240)*V], TP86 [*fkb-4(ok240)*V*; fkb-5(tm475)*I] and TP83 [*fkb-3*(*tm348*) *fkb-4*(*ok240*)V; *fkb-5*(*tm475*)I] were made by performing standard genetic crosses with the relevant strains. Strains were genotyped for the deletion alleles using a multiplex single worm PCR strategy.

The cold-sensitive assays were performed by moving L4 larvae or young adults grown at 15-20°C to 12°C and observing the development of their progeny. In addition, L1 larvae were temperature shifted in a similar manner and observed.

The cuticle integrity assays were performed on strains maintained at 12°C and 20°C by picking groups of 10 L1 larvae into 100µl of the test solutions (M9 balanced salts, distilled water and 1% mercaptoethanol in distilled water) in a 96-well microtitre plate. After 10 minutes the larvae were viewed under a dissecting microscope and scored for cuticle integrity, as assessed by cuticle rupture and release of internal body contents.

Construction of C. elegans fkb-3, fkb-4 and fkb-5 promoter reporter gene fusion; spatial analysis- The *fkb-3*, *-4* and *-5* reporter gene plasmids were constructed using the *C. elegans lacZ* promoterless reporter gene expression vector pPD95.03 or pPD96.04 (Addgene). Reporter gene constructs comprised 1.5-2 kb of the potential upstream regulatory region and part of the first exon (or part of the first and second exons in the case of *fkb-4* construct 2) fused in a translationally in-frame context to the *lacZ* reporter gene. Constructs were generated from genomic DNA template by PCR with the following primers: *fkb-3* construct (from –2017 to +5 relative to the ATG),

fkb3pF(HindIII) 5' gcgcaagcttgaactgttctcaagggaagatggg 3' and fkb-3pR (BamHI) 5' gcgggatccagcatttttgtgctggttcggtttag 3'; fkb-4 construct 1 (from –1966 to + 23 relative to the ATG), fkb4p1F(XbaI) 5' gatctagatacgggtgtccatatacagaac 3' and fkb4p1R (BamHI) 5′ gccggatccgtggttcgatataaaaacttcatttg 3'; *fkb-4* construct 2 (from –1269 to + 284 relative to the ATG), fkb4p2R (SalI) 5' gcggtcgacgaggctcatatgatcaggatagtg 3' and fkb4p2R (BamHI) 5' gcgggatcctcgctctcatctattgacgatg 3'; *fkb-5* construct (from –1716 to + 86 relative to the ATG), fkb-5pF(PstI) 5′ gcgctgcagttccaacaagctcatctggtcctg 3′ and fkb-5pR (BamHI) 5′ gcgggatcccatcctcatccttccattgaagc 3′. The resulting clones were digested with the appropriate combination of restriction enzymes and ligated into similarly digested pPD95.03 or pPD96.04. For each construct, nematode transformation was performed by microinjection of plasmid DNA into the gonad syncitium using standard methods as described (Mello and Fire, 1995). Each construct was independently injected at 15 µg/ml (also at 100 µg/ml for *fkb-4* construct 2) along with the following phenotypic markers (at 100 µg/ml); *rol-6* into a wild type background or *unc-76* rescue plasmid into an *unc-76* (DR96) mutant background. Transformed nematodes were identified either by the mutant roller phenotype or a rescued Unc-76 phenotype and at least three transgenic lines were established for each construct. The semi-stable transmitting lines were fixed and stained for β-galactosidase activity using standard methods (Fire, 1992). Sensitive staining procedures using ten-fold higher substrate concentrations were applied to the *fkb-4* lines that were negative using the standard method.

Transgenic rescue of cold-sensitive lethal mutants- Rescue of TP86 (*fkb-4; fkb-5*) and TP83 (*fkb-3; fkb-4; fkb-5)* deletion mutants with transgenic *fkb-4* or *fkb-5* was performed by microinjection. The *fkb-4* rescue clone was generated by PCR on N2 genomic DNA with primers Fkb-4 NPF and Fkb-4 Resc R 5' cactttcaaggcgagttacgttc 3' using *Pfu* DNA polymerase (Stratagene). The 3562 bp product was cloned into pCR2.1TOPO. For the *fkb-5* clone, the primers Fkb-5 PF and Tm475 R1 generated a 3226 bp product that was likewise cloned into pCR2.1TOPO. All injections were carried out at 10 µg/ml rescue construct plus 5 µg/ml *dpy-7*::GFP and 135 µg/ml pBluescript SKM (Stratagene). Transgenic lines were established by expression of co-injected GFP marker, maintained at the permissive temperature of 20°C then 5 to 10 transgenic animals were shifted to 12°C. Controls including the original strains TP86 and TP83 were also shifted and assessed microscopically.

Temporal analysis by semi-quantitative RT-PCR- The semi-quantitative RT-PCR method including the generation of synchronous nematode cultures for staged mRNA and subsequent cDNA synthesis are described in detail elsewhere (Johnstone and Barry, 1996). The gene combinations (*fkb-*

3, -4 and *-5* in combination with the control gene *ama-1*) were amplified from the cDNA samples representing staged mRNA that corresponded to two hourly intervals during post-embryonic development. The primers used were as follows: *fkb-3*, fkb3rtF 5' ggagtctggagatcaattggag 3' and fkb3rtR 5' gagctctggtggaataacaac 3'; *fkb-4*, fkb4rtF 5' tgagaagggagttgaaattgga 3' and fkb4rtR 5' tctccttcacacattccatcc 3'; *fkb-5*, fkb5rtF 5' gccgaggagcaaaagcttcaatgg 3' and fkbrtR 5' ttacaactcgtcgcgttggatgagc 3'; *ama-1*, ama1F 5' ttccaagcgccgctgcgcattgtctc 3' and ama1R 5' cagaatttccagcactcgaggagcgga 3'. The PCR reactions were electrophoresed, Southern blotted and probed with *fkb-3, fkb-4* or *-5* PCR products labelled with [α-^{32}P]dCTP using random priming (Prime-It®II Random Primer Labelling Kit, Stratagene), in combination with similarly labelled *ama-1* PCR products. The blots were autoradiographed using a Typhoon imager (Molecular Dynamics), and bands representing the respective genes were quantified using ImageQuant 5.1 software (Molecular Dynamics). The relative abundance of the individual *fkb* genes were determined by comparing the signal to *ama-1*. At least three sets of PCR reactions with subsequent quantifications were performed in order to accurately determine the relative abundance of the individual *fkb* genes to *ama-1*.

Recombinant protein expression and PPIase assay on FKB-3- fkb-3 was cloned from wild type *C. elegans* mixed stage cDNA by PCR using the following primers: FKB3F 5' ccggaattccgcaaatgaccgttcatggacc 3' and FKB3R 5' cctctagactagagttcctcttttcctggtc 3', digested with *Eco* RI and *Xba* I and ligated into similarly cut pMalc2 vector (New England Biolabs) prior to transformation into XL1 expression cell lines. Maltose binding fusion proteins were produced following the manufacturer's instructions and then digested with factor-Xa (New England Biolabs). The affinity purified recombinant FKB-3 was assayed for PPIase activity following published protocols (Kofron *et al.*, 1991; Page *et al.*, 1996). This assay determines the rate of conversion of *cis* to *trans* of a proline-containing peptide substrate N-succinyl-Ala-Leu-Pro-Phe-p-nitroanilide (Bachem). The kinetic parameters (k_{cat}, K_m and k_{cat}/K_m) were calculated using spreadsheet software (Microsoft Excel).

Immunocytochemistry of C. elegans embryos, larvae and adults - DPY-7 and MH27, the respective collagen and seam cell boundary specific monoclonal antibodies were applied to freeze-cracked embryos, L1 larvae and adults of the various strains described according to published method (Rogalski *et al.*, 1993). Briefly, the washed samples were freeze cracked on poly L-lysine slides, blocked in 3% BSA, washed, and the anti-DPY-7 (McMahon *et al.*, 2003) or anti-MH27 monoclonal antibodies (Francis and Waterston, 1991) added at 1/50 dilutions. Following incubation, the slides

were washed and the anti-mouse conjugate secondary antibody Alexa Fluor 488 (Molecular probes) was applied at 1/100 dilution. Following incubation, the slides were washed and viewed by microscopy.

Microscopy- All samples were viewed either via DIC or epifluorescence on a Zeiss Axioskop 2 microscope and images were taken with a Zeiss MrS digital camera.

Results

The secretory pathway associated FK506-binding proteins of C. elegans. Examination of the completed genome sequence of *C. elegans* has uncovered eight distinct FK506-binding protein-encoding genes, termed *fkb-1* to *-8* (Bell, 2006 #424). Of this family of proteins, FKB-3, -4 and -5 were found to possess both secretory signal peptides as assessed by SignalP (expasy.ch) and have C-terminal endoplasmic reticulum retention signals; RDEL or KEEL (Figure 1 A). In addition, these FKBs encode proteins of 27-29 kDa that have two consecutive FK506 peptidyl prolyl isomerase domains separated by a 25-27 amino acid linker sequences. The three FKBs also share a high level of amino acid identity; 54% between FKB-3 and -4; 59% between FKB-3 and -5, 66% between FKB-4 and -5. From the aforementioned features, *fkb-3, -4* and *-5* were expected to encode secretory pathway catalysts and so were functionally characterized individually and in combination.

FKB-3 recombinant protein has peptidyl prolyl cis-trans isomerase activity. FKB-3 was cloned and expressed as a maltose binding protein fusion, cleaved by factor-Xa and examine for PPIase activity against a synthetic Leu-Pro containing synthetic substrate following standard PPIase assay conditions (Kofron *et al.*, 1991; Page *et al.*, 1996). This recombinant protein can actively convert Leu-Pro *cis* to Leu-Pro *trans* and thereby release the p-nitroanalide chromophore. The activity was calculated as;
k_{cat} 110 ± 34 s^{-1}, K_m 581 ± 88 mM, and k_{cat} / K_m 0.189 ± 0.039 × 10^6 M^{-1} s^{-1}, and this is comparable to previously published data for bovine FKB
(k_{cat} 344 ± 26 s^{-1}, K_m 520 ± 85 mM, and k_{cat} / K_m 0.66 ± 0.12 × 10^6 M^{-1} s^{-1})(Kofron *et al.*, 1991).

FKB-3 and FKB-5 are expressed in the collagen synthesizing hypodermis. The putative promoter regions of the *fkb* genes *fkb-3*, *-4* and *-5* were cloned into β-galactosidase reporter constructs in an attempt to elucidate their spatial expression patterns (regions depicted in Figure 1 B). These vectors contain a multi-intron reporter gene to enhance expression and a SV40 nuclear localization signal that aids the identification of the cells expressing the transgene. All three

constructs included the translational start of the first exon and produced transgenic lines following gonadal injection. A predominantly hypodermal cell expression pattern was displayed in the *fkb-3* and *-5* lines. The hypodermis is the collagen synthesising tissue, which in turn performs many essential functions in the nematode, the most important of which being the synthesis and secretion of the cuticular exoskeleton. All lifecycle stages examined from mid- embryo through larval to adult stage displayed this hypodermal expression pattern (Figure 2). Both *fkb-3* and *fkb-5* share very similar expression patterns that included the lateral seam cells, tail and head hypodermal cells (Figure 2). The observed expression pattern was consistent between the transgenic markers applied, namely hypodermal *rol-6* and neuronal Unc-76 rescue. Transgenic lines derived from the *fkb-4* injection failed to produce a detectable expression pattern following β-galactosidase staining. A second construct was therefore assessed that included the first intron and part of the second exon of *fkb-4*, but again no detectable expression pattern was observed following the increased concentration of the injection construct or by applying sensitive staining methods. These observations indicated that the *fkb-4* promoter regions examined generated transgenic expression that was at a level below the sensitivity of this reporter assay.

The temporal expression pattern of the secreted FKBs is consistent with a role in collagen biogenesis. Semi-quantitative RT-PCR was carried out to determine the expression profile of the secreted FKBs throughout the nematode's post-embryonic lifecycle. The signal of the individual transcripts were measured at two hour time-points throughout larval and early adult development and were standardized by comparing them to the abundance of the constitutively expressed gene *ama-1*, encoding the large RNA polymerase subunit (Bird and Riddle, 1989). These experiments were repeated in triplicate and standard deviations were plotted for each *fkb* gene (Figure 3). It is apparent from this study that *fkb-3* and *fkb-5* in addition to sharing a spatial expression pattern also share a similar temporal expression pattern, with both transcripts having peaks of abundance that occur at the inter-molt period for each larval stage and drop off during the actual molt and in the adult stage (Figure 3). *fkb-5* has a higher overall abundance than *fkb-3* that peaks two hours prior to *fkb-3* in the earliest larval stages, namely the first and second larval stages (Figure 3). This oscillating abundance coincides with cuticle collagen synthesis and has been observed previously for numerous cuticle collagen genes including, *sqt-1, sqt-3* and *dpy-13* (Johnstone and Barry, 1996) and their biosynthetic enzymes, including *dpy-18, phy-2* and *pdi-2* (Winter and Page, 2000). The *fkb-4* transcript was detectable by RT-PCR but at a level lower than either *fkb-3* or *-5* (Figure 3). A steady-state but low

level of expression was observed in all larval stages and in the early adult stage but became undetectable in the mature adult stage.

FKB deletion mutant analysis reveals a combined cold-sensitive lethal effect. Deletion mutants in *fkb-3*, *fkb-4* and *fkb-5* were screened for or were obtained from the ongoing genome-wide deletion mutant consortia. The positions of the deletions were sequenced and mapped (regions depicted in Figure 1B). Double and triple *fkb* deletion mutants were made by standard genetic crosses, which were confirmed by single worm PCR (result not shown). The progeny of single and combined mutants were assessed for growth and morphology defects at a range of temperatures from 12°C to 25°C (Table 1). Wild type, N2 strain nematode grow slowly but develop otherwise normally at 12°C. All single mutant strains were phenotypically wild type at all temperatures tested (Table 1). In addition, the *fkb-3; fkb-4* double (TP81) and *fkb-3; fkb-5* doubles (TP60) were wild type at all temperatures examined. TP60 did however generate an extremely low level of Dumpy (Dpy, short and fat) mutants, but only at 12°C. The two independent *fkb-4(ka4); fkb-5(tm475)* and *fkb-4(ok240); fkb-5(tm475)* double mutant strains (TP73 and TP86, respectively) displayed a complete larval lethal phenotype at 12°C but remained wild type at 15°C to 25°C (Figure 4). The *fkb-3; fkb-4*; *fkb-5* triple mutant (TP83) likewise displayed a completely penetrant larval lethal phenotype at 12°C (Table 1, Figure 4). The TP73, TP86 and TP83 mutants all grew slowly at 12°C and failed to proceed beyond the first larval stage (Figure 4). The mutant L1 larvae were shorter and slightly dumpier than the wild type worms and many also exhibited a "baggy" cuticle molt defect (Figure 4, E and F). The double (TP86) and triple (TP83) mutants also produced a range of very severe body morphology defects at 12°C, both in the unhatched and newly hatched L1 larvae, including severe Dpy and coiled phenotypes (Figure 7, C and E; Figure 8, A and C).

The basis of this synthetic cold-sensitive lethal phenotype was examined in more detail by further characterizing the TP83 triple and TP86 double mutant strains. A maternal contribution to this effect was ruled out following the examination of TP83, TP86 and TP73 embryos derived from hermaphrodite mothers grown at 20°C, then hatched and maintained on plates at 12°C, as all strains remained larval lethal. However, when embryogenesis was completed at 15 or 20°C and the hatched L1s were transferred to 12°C, the TP83 and TP86 mutant strains were no longer larval lethal (Figure 5). These mutants could now develop to adulthood, however the larval and adult stages developed slowly and were smaller in size compared to identically treated wild type worms (compare Figure 5 A with C and E, and 5 B with D and F). This result confirmed that the cold-sensitive lethal effect was

occurring during embryogenesis, presumably during the synthesis of the first larval cuticle. The ability of the TP83 triple mutant strain to enter and recover from the arrested dauer larval stage at 20^{o}C was also examined and found to be comparable to wild type.

Complementation of the cold-sensitive larval lethal phenotype by fkb-4 or fkb-5. The triple mutant strain TP83 and the combined *fkb-4; fkb-5* double (TP86) were transformed with either a wild type copy of the *fkb-4* or the *fkb-5* gene in combination with a *dpy-7::gfp* transformation marker. Semi-stable transgenic lines were established and L4 stage larvae were transferred to plates at the non-permissive temperature and their progeny were assessed for viability. Both *fkb-4* and *fkb-5* were able to fully rescue the cold-sensitive larval lethal phenotype at 12°C, with rescued nematodes developing normally through larval to egg-laying adult stages (Figure 6). An upstream region of the adjacent *C. elegans* gene C50F2.5 was also removed by the *fkb-5* allele *tm475* and a large section of ZC455.9 coding sequence is likewise deleted by *fkb-4(ok240)*. The involvement of these genes was excluded following transgenic rescue, as both constructs would not have reintroduced functional copies of the adjacent genes (Figure 1 B).

Analysis of the additional secretory FKBs. The remaining secretory pathway FKBs namely, FKB-1 and FKB-7 were analysed to determine if they were expressed in the same tissue and if they can interact with *fkb-3*, *-4* and *-5* through reporter/promoter construct examination and via RNA interference studies, respectively. *fkb-1* encodes a small 13 kDa single-domain FKB that found to be expressed exclusively in the gut , whereas *fkb-7* represents a 35 kDa single domain FKB that is expressed in the nervous system (data not show). RNA interference performed at 15°C and 20°C with either *fkb-1* or *fkb-7* in the *fkb*-triple mutant background produced no additional effects (data not shown).

Cuticle collagen and underlying seam cell disruption in cold-sensitive mutants. The cuticle and the underlying hypodermis of the double and triple mutants were examined following immunolocalization of cuticle collagen-specific antibody (DPY-7) and a seam cell boundary-specific antibody (MH27) at the non-permissive temperature. In wild type embryos the DPY-7 antibody is retained within the cell in a perinuclear ER-associated location prior to elongation. Once the first larval cuticle is laid down at elongation the DPY-7 epitope is then arranged in regular rings corresponding to the circumferential folds of the cuticle (McMahon *et al.*, 2003). This annular ring localization is found in all larval and adult stage cuticles (Figure 7 J). The pre-elongated embryos of the triple (Figure 7 B) and double mutants (not shown) display the wild-type DPY-7 perinuclear

localization. Following elongation the expression pattern of DPY-7 becomes extremely patchy and severely disrupted prior to (Figure 7 D) and following hatching (Figure 7 F and H). The severity of DPY-7 disruption correlates to the severity of the morphological disruption noted in these L1 mutants (compare Figure 7, H and F). The antibody MH27 is associated with the apical epithelial cell gap junctions and provides an excellent marker for the hypodermal seam cells that are arranged in a regular fashion along opposing lateral sides of the wild type nematode (Figure 8 H). MH27 expression is extremely disrupted and patchy in newly hatched triple and double mutants (Figure 8 B and D) and in the arrested L1 larvae grown at 12°C (Figure 8 F). As described for DPY-7, there is a direct correlation between the severity of morphological disruption and associated MH27 disruption. The above observations indicate that the combined *fkb* deletions are having affects on both the cuticle structure and the underlying hypodermis at the non-permissive temperature.

The triple mutant TP83 affects the structural integrity of the cuticle and is temperature dependant. The L1 first stage larvae from the triple mutant strain were maintained at both the permissive (20°C) and the non-permissive (12°C) temperatures, then placed in various osmotic or reducing solutions before being assessed for the associated effects on cuticle integrity (Table 2). Wild type L1 nematodes were completely unaffected following a 10 minute exposure to isotonic buffer (M9 salt solution), distilled water or mild reducing conditions (1% β-mercaptoethanol). Likewise, TP83 triple mutants were unaffected in the isotonic M9 buffer at either temperature. Exposure to osmotic stress, in the form of distilled water resulted in 20% and 26% of the nematodes bursting at 20°C and 12°C, respectively. The mild reducing conditions of 1% mercaptoethanol for 10 minutes, had a significant effect at 20°C causing 18% of the worms to explode. A more dramatic affect was however observed at 12°C, as more than half (62%) of the larvae exploded and extruded their internal contents (Table 2).

Discussion

There are eight genes which encode FK506-binding proteins in the *C. elegans* genome (Bell *et al*., 2006), three of which encode 28 to 29 kDa secretory proteins with dual PPIase domains and endoplasmic reticulum retention signals. The proteins, FKB-3 and FKB-5 are spatially expressed in the collagen synthesizing hypodermal tissue positioning them in the correct subcellular compartment for involvement in collagen biogenesis. No spatial expression pattern has so far been defined for *fkb-4*, however the promoter region selected for these studies was also used to successfully rescue the

cold-sensitive triple mutant, indicating that it is sufficient to generate expression but at a level undetectable in the reporter assays. The genetic interactions described between *fkb-4* and *-5* suggests that *fkb-4* must also be expressed in the hypodermis. The temporal expression pattern confirmed that *fkb-4* is expressed at a low level while *fkb*-3 and *fkb*-5 are expressed in cycles of abundance that correspond to the cuticle collagen synthesis cycle. Previous experiments have indicated that *fkb-3, -4* and *-5* are differentially expressed between wild type and *daf-2*, dauer mutant background and confirmed that *fkb-4* was expressed at an extremely low level (Yu and Larsen, 2001). In addition to the secreted *fkb*s, a number of cuticle collagen genes were also differentially regulated by *daf-2* (Yu and Larsen, 2001).

As would be predicted, we have demonstrated that one of the secreted FKBs has potent PPIase activity against a synthetic substrate that was comparable to FK506-binding proteins from other species (Kofron *et al.*, 1991). FKB-4 and -5 would be expected to be similarly active. The functional significance of these gene products with respect to folding the cuticle collagens and the formation of a proper cuticle was established following the examination of combined deletion mutants. A combination of all three genes or of *fkb-4* and *fkb-5* together led to a subtle synthetic phenotype, namely a cold-sensitive lethal phenotype. Deletion mutant embryos reared at 12°C hatched successfully but failed to complete larval development. In addition, the mutant larvae exhibited irregular cuticle collagen expression, their underlying hypodermis was disrupted and their cuticles were structurally weakened, being more liable to explode when exposed to mild reducing conditions. In contrast, nematodes developed normally at the permissive temperatures of 15-25°C, exhibited normal collagen localization and, following exposure to reducing agents, displayed only a slight weakening of the cuticle. Accordingly, mutant nematodes exposed to the non-permissive temperature during their larval development are viable and predominantly wild type in appearance.

The free-living nematode *C. elegans* is found in anthropogenic locations throughout the world, being commonly isolated from gardens and compost heaps where it feeds on the associated microorganism-rich organic material. In these locations the predominant form is the dauer or arrested development larval stage, with mature adult stages requiring ideal environmental conditions namely food, temperature and available oxygen (Kiontke and Sudhaus, 2006). Numerous experimentally-derived, temperature-sensitive mutants have been characterised in *C. elegans*, the majority of which are restricted at 25°C, with cold-sensitive mutants being relatively rare. Other cold-sensitive mutants however do include the *wrt-1* hedgehog related signalling gene, that when mutated results in

embryonic and larval death in conjunction with morphological and molt defects (Hao *et al*., 2006). It may be hypothesised that the secretory pathway *fkb* genes are relatively redundant and dispensable at normal physiological temperatures (15-22°C) but are absolutely required at sub-physiological temperatures. This most probably relates to the role that FKBs play in the rate-limiting isomerisation of proline bonds, in particular the proline-rich cuticle collagens that are abundantly synthesized in repeated waves that correspond to the molting cycle. It is well established that proline isomerisation and proper protein folding occurs in the absence of these catalysts due to thermal isomerization (Bächinger, 1987 #10; Kofron, 1991 #518; Wang, 2005 #522} and therefore removing the thermal isomerization by decreasing the physiological temperature necessitates the requirement for the PPIase activity of the FKBs. In corroboration of this, a recent study has identified a FKBP member from the psychrotophic bacteria *Shewanella spp* that is overexpressed at 4°C compared to 20°C, and it has been hypothesized to be involved in cold adaptation, aiding the proper folding of proteins at low temperatures (Suzuki *et al*., 2004).

The specific function of secretory pathway FKBs in development was previously challenged following combined *cyp* and *fkb* knockout experiments in yeast (Dolinski *et al*., 1997). The combined immunophilin yeast knockout was viable and the authors therefore concluded that each CYP and FK506-binding protein interacts with a unique set of proteins and performs a distinct but non-essential function (Dolinski *et al*., 1997). In metazoans this may not necessarily be the case, and there are several examples where secreted CYPs are involved in specific functions. The best characterized example being the specific folding of the photoreceptor rhodopsin by the *ninaA* cyclophilin in *Drosphila melonogaster* (Schneuwly *et al*., 1989; Stamnes *et al*., 1991). In addition, the secreted vertebrate cyclophilin B isoform has been established as assisting the proper folding and exit of type I procollagen from the ER (Smith *et al*., 1995). Cyclophilin has also been shown to accelerate the *in vitro* refolding of type III vertebrate collagens (Bächinger, 1987) and the inhibitor cyclosporin A effectively decreases the *in vivo* folding of type I procollagens (Steinmann *et al*., 1991), presumably through prolyl isomerase inhibition. In addition to having a high proline-content, the non-globular collagens fold in a progressive fashion requiring each proline *cis-trans* isomerisation to be completed before triple helix formation can proceed (Bächinger, 1987). The FKB family are by comparison less well characterized than the CYPs. FKBP65 however, represents a secretory pathway FKB that is located in the ER and is involved in ECM formation via its association with tropoelastin (Patterson *et*

al., 2000) and in addition, this PPIase has also been shown to assist in the folding of vertebrate type III collagen (Zeng *et al*., 1998).

It can be envisaged that under normal physiological temperatures nematodes develop in a conventional manner, synthesizing waves of cuticle collagens that are in turn secreted and assembled into a complex ECM in the presence but not the dependence of active PPIase enzymes. Under conditions of cold stress however these enzymes are essential, being required to chaperone or indeed fold the collagens that must be expressed in the correct temporal, spatial, quantitative and qualitative fashion to allow the proper folding, secretion and assembly of the nematode cuticle components. The cuticle collagens represent 1% of the entire genome of *C. elegans*, comprising more than 170 genes (Page and Winter, 2003). There is enormous complexity in the expression and association of these collagens that is only just beginning to be uncovered. Individual cuticle collagens can be stage-specific and indeed there can also be additional complexity in individual collagen expression within each larval stage. It is predicted that the evolutionary conserved, ubiquitous PPIase enzymes may perform essential overlapping functions in multicellular animals including the chaperoning and catalysis of protein folding events, particularly collagen biogenesis in the natural environment.

Acknowledgments

The *fkb-3*(*tm348*) and *fkb-5(tm475)* deletion alleles were produced by the National Bio-Resource Project for the Nematode (Japan) and the *fkb-4*(*ok240*) deletion by the *C. elegans* Gene Knockout Consortium (Oklahoma). The *dpy-7::*GFP plasmid and DPY-7 antibody were gifts from Iain Johnstone (Glasgow) and the *Caenorhabditis* Genetic Centre provided some of the nematodes strains used. This work was funded by the Medical Research Council UK through a Senior Fellowship award to A.P.P. (G117/476).

Figure Legends

Figure 1. A. Amino acid alignment of FKB-3, -4 and -5 via Clustal W. The position of the dual PPIase domains are indicated by arrowheads; black for FKB-3, grey for FKB-4 and white for FKB-5; ER retention signals are highlighted in bold. B. Schematic representation of the gene structure of *C. elegans* FKB encoding genes *fkb-3, -4* and *-5*. Black boxes depict coding exons with arrows indicating the translational orientation of the gene. Adjacent, non-related genes are depicted by open boxes. Solid lines labelled (P) represent the promoter regions use in reporter construct and (Res) are the rescue constructs. The positions and lengths of the associated deletions are depicted by shaded boxes.

Figure 2. Spatial expression pattern of the most abundant ER resident FKBs. Nuclear localized message depicted following β-galactosidase staining of *lac*Z::promotor expressing transgenic nematodes. A. L1 stage larva expressing *fkb-3*::*lac*Z transgene in the hypodermal cell nuclei. B. Adult stage hermaphrodite expressing *fkb-3*::*lac*Z transgene in the hypodermal cell nuclei. C. L1 stage larva expressing *fkb-5*::*lac*Z transgene in the hypodermal cell nuclei. D. Adult stage hermaphrodite expressing *fkb-5*::*lac*Z transgene in the hypodermal cell nuclei.

Figure 3. Semi-quantitative RT-PCR examination of temporal expression pattern of *fkb-3, fkb-4* and *fkb-5* throughout the post-embryonic lifecycle of *C. elegans*. Relative transcript levels were assessed in triplicate by comparing the abundance of the test gene compared to that of the constitutively expressed RNA polymerase gene *ama-1*. cDNA samples represent two hour intervals proceeding the initiation of feeding in starved L1 larvae. L1 to L4 depict larval stages.

Figure 4. Morphological comparison of combined *fkb* deletion mutants to wild type nematodes grown at 12ºC for their entire lifecycle. A. Depicts adult stage wild type (N2) hermaphrodite raised at 12°C (size is 970µm). B. L1 stage N2 larvae raised at 12°C (sizes are 250 and 280µm). C. TP83 (*fkb-3/fkb-4/fkb-5* triple mutant) arrested L1 raised at 12°C (size 190µm). D. TP81 (*fkb-3/fkb-4* double) viable L2 raised at 12°C. E. High magnification image of TP83 (*fkb-3/fkb-4/fkb-5* triple) arrested L1 larvae raised at 12°C (size 200µm). F. High magnification image of TP86 (*fkb-4/fkb-5* double) arrested L1 larvae raised at 12°C (size 230µm). Scale bar in A is 100µm; B, C and D are 50µm; E and F are 20µm.

Figure 5. Morphological comparison of combined *fkb* deletion mutants to wild type nematodes grown at the non-permissive (12ºC) temperature during their post-embryonic lifecycle. All nematodes were exposed to non-permissive temperature as L1 larvae. A. Depicts a typical L4 stage wild type (N2) raised at 12°C for 5 days (size is 860µm). B. Adult N2 raised at 12°C for 7 days (size 1200µm). C. TP83 (*fkb-3/fkb-4/fkb-5* triple mutant) L3 raised at 12°C for 5 days (size 370µm). D. TP83 (*fkb-3/fkb-4/fkb-5* triple mutant) adult raised at 12°C for 7 days (size 900µm). E. TP86 (*fkb-4/fkb-5* double mutant) L3 raised at 12°C for 5 days (size 350µm). F. TP86 (*fkb-4/fkb-5* double mutant) adult raised at 12°C for 7 days (size 860µm). Scale bars represent 100µm.

Figure 6. Rescue of cold-sensitive larval lethal phenotype of TP83 (*fkb-3/ fkb-4/fkb-5)* triple mutants raised at non-permissive temperature (12°C). A. Depicts a DIC image of TP83 mutant rescued following injection of a wild type copy of *fkb-4* gene. B. U.V image of A, depicting co-expression of *dpy-7*::GFP transformation plasmid. C. Depicts a DIC image of TP83 (*fkb-3/ fkb-4/fkb-5* triple mutant) rescued following injection of a wild type copy of *fkb-5* gene. D. U.V image of C, depicting co-expression of *dpy-7*::GFP transformation plasmid. All Images taken at identical magnification and depict adult hermaphrodites.

Figure 7. Cuticle collagen expression in multiple *fkb* mutants maintained at 12°C as assessed through DPY-7 expression pattern. A. TP83 (*fkb-3/fkb-4/fkb-5* triple mutant) pre-elongate embryo depicting normal morphology via DIC. B. Epifluorescence image of A revealing normal ER-restricted DPY-7 hypodermal expression pattern. C. TP86 (*fkb-4/fkb-5* double mutant) elongated embryo exhibiting

abnormal DIC morphology. D. Epifluorescence image of C depicting associated aberrant and patchy DPY-7 expression. E. Newly hatched TP83 L1 larvae depicting the severe Dpy phenotype, DIC image. F. Epifluorescence image of E depicting severely disrupted DPY-7 expression. G. Mutant phenotype of TP86 L1 larvae visualised by DIC. H. Epifluorescence image of G depicting disrupted DPY-7 hypodermal expression pattern. I. DIC image of wild type adult cuticle, visualised by DIC. J. Epifluorescence image of I depicting the wild type annular pattern of DPY-7 expression in the adult cuticle. A- H scale bar represents 50μm; I and J are 20μm.

Figure 8. Examination of hypodermal seam cell morphology in multiple *fkb* mutants maintained at 12°C, as assessed through MH27 antibody expression pattern. A. TP83 (*fkb-3/fkb-4/fkb-5* triple mutant) elongate embryo at hatching displaying severe Dpy and coiling phenotypes, viewed by DIC. B. Epifluorescence image of A revealing associated severe disruption to the seam cells. C. TP86 (*fkb-4/fkb-5* double mutant) newly hatched severely mutant L1, viewed by DIC. D. Epifluorecence image of C depicting aberrant MH27 expression pattern and disrupted irregular seam cells. E. TP86 L1 larvae depicting Dpy phenotype as assessed by DIC. F. Epifluorescence image of E depicting disrupted MH27 antibody expression pattern. G. DIC image of wild type L1. H. Epifluorescence image of G depicting the wild type regular expression pattern of MH27 in the hypodermis. All images taken at identical magnification, scale bars represent 50μm.

Table 1. Temperature-dependant phenotypes of single and combined *fkb* mutants.

Strain	Genotype	12°C	15°C	20°C	25°C
TP8	*fkb-3(tm348)*V	w.t.	w.t.	w.t.	w.t.
RB1213	*fkb-4(ok240)*V	w.t.	w.t.	w.t.	w.t.
TP65	*fkb-4(ka4)*V	w.t.	w.t.	w.t.	w.t.
TP9	*fkb-5(tm475)*I	w.t.	w.t.	w.t.	w.t.
TP81	*fkb-3(tm348) fkb-4(ok240)*V	w.t.	w.t.	w.t.	w.t.
TP60	*fkb-3(tm348)*V*; fkb-5(tm475)*I	w.t.[a]	w.t.	w.t.	w.t.
TP73	*fkb-4(ka4)*V*; fkb-5(tm475)*I	Mutant	w.t.	w.t.	w.t.
TP86	*fkb-4(ok240)*V*; fkb-5(tm475)*I	Mutant	w.t.	w.t.	w.t.
TP83	*fkb-3*(*tm348*); *fkb-4*(*ok240*)V; *fkb-5*(*tm475*)I	Mutant	w.t.	w.t.	w.t.

[a] Low level of slight Dpy phenotype observed. W.t., wild type.

Table 2. Effects of osmotic stress on wild type and triple *fkb* mutants.

Strain	Temp.	Genotype	Number of ruptured L1 larvae		
			M9	dH_2O	1% βme
N2	20°C	wild type	0/41	0/40	0/38
TP83	20°C	*fkb-3*Δ *fkb-4*Δ*; fkb-5*Δ	0/40	8/39	7/38
N2	12°C	wild type	0/38	0/40	0/37
TP83	12°C	*fkb-3*Δ *fkb-4*Δ*; fkb-5*Δ	0/45	12/46	28/45

M9, isotonic salt solution. βme, β-mercaptoethanol reducing solution.

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Figure 1A

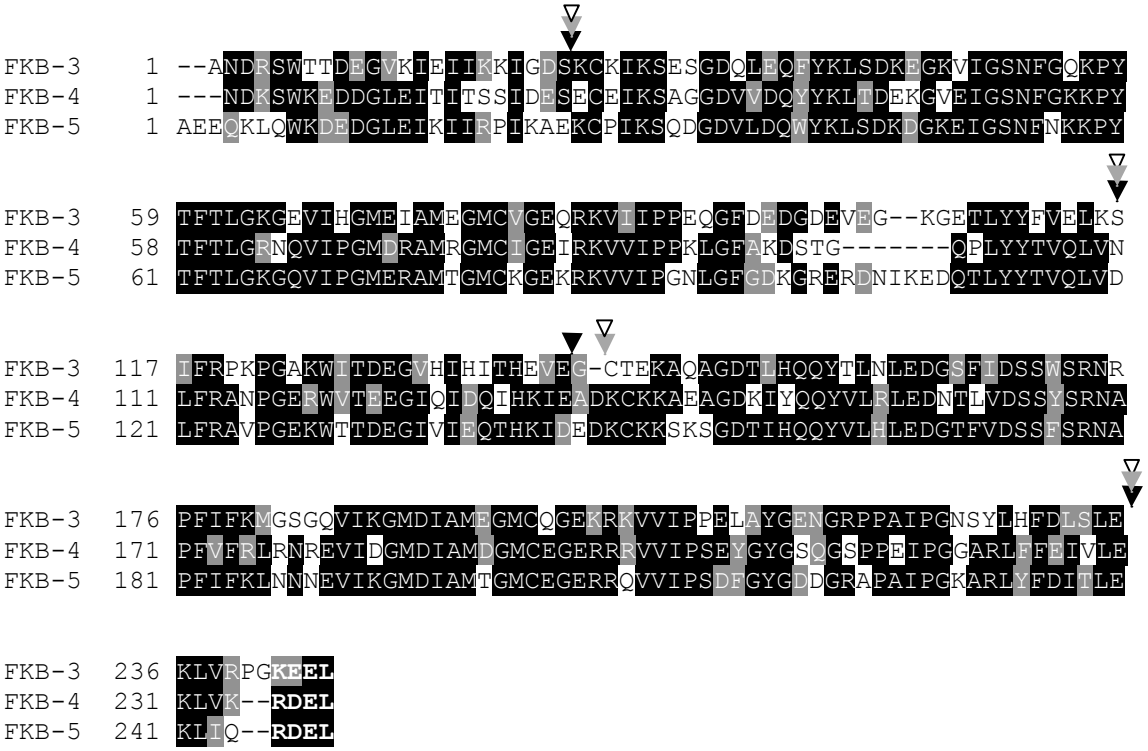
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Figure 1B

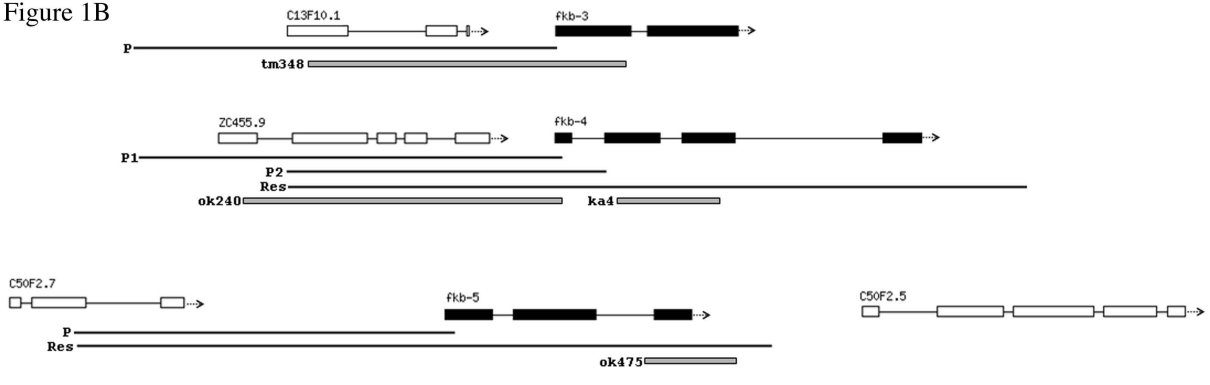
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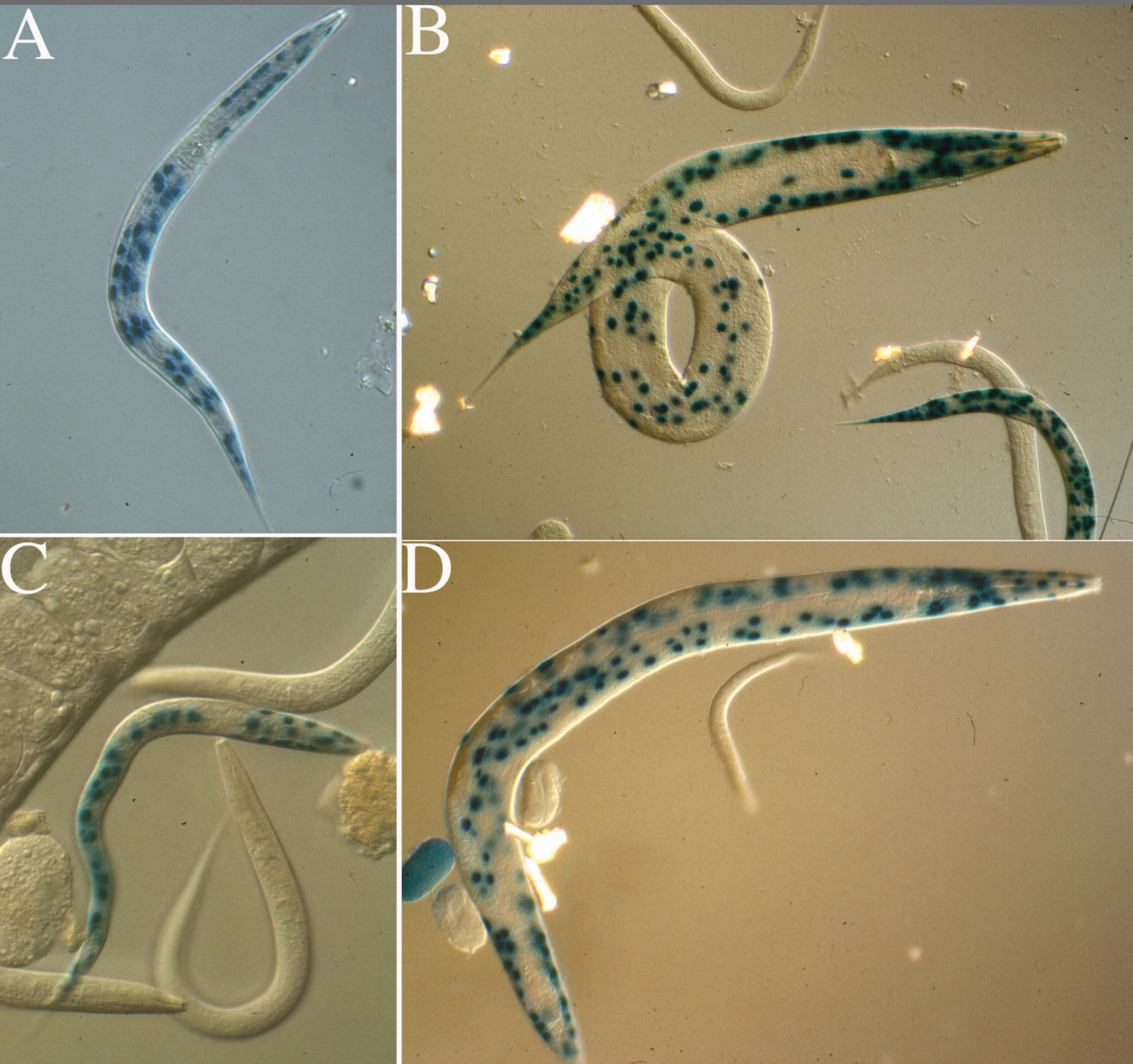
Figure 2



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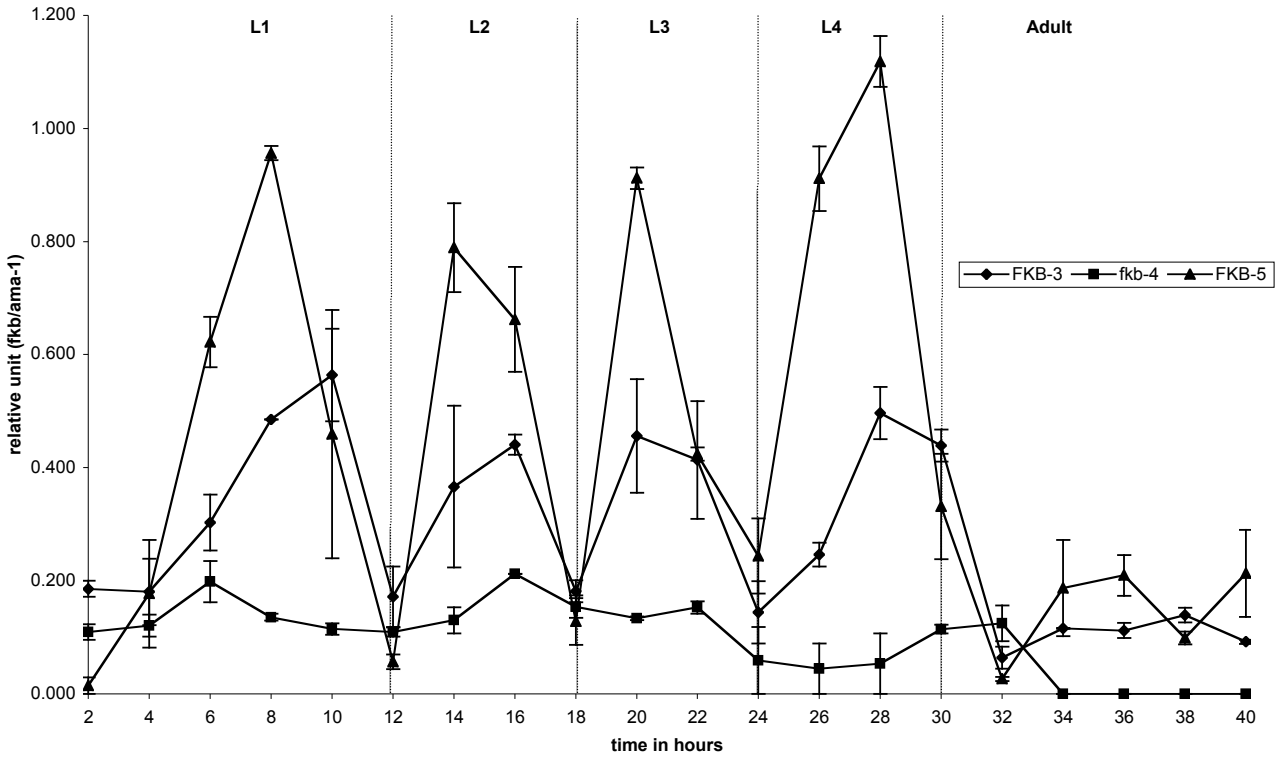
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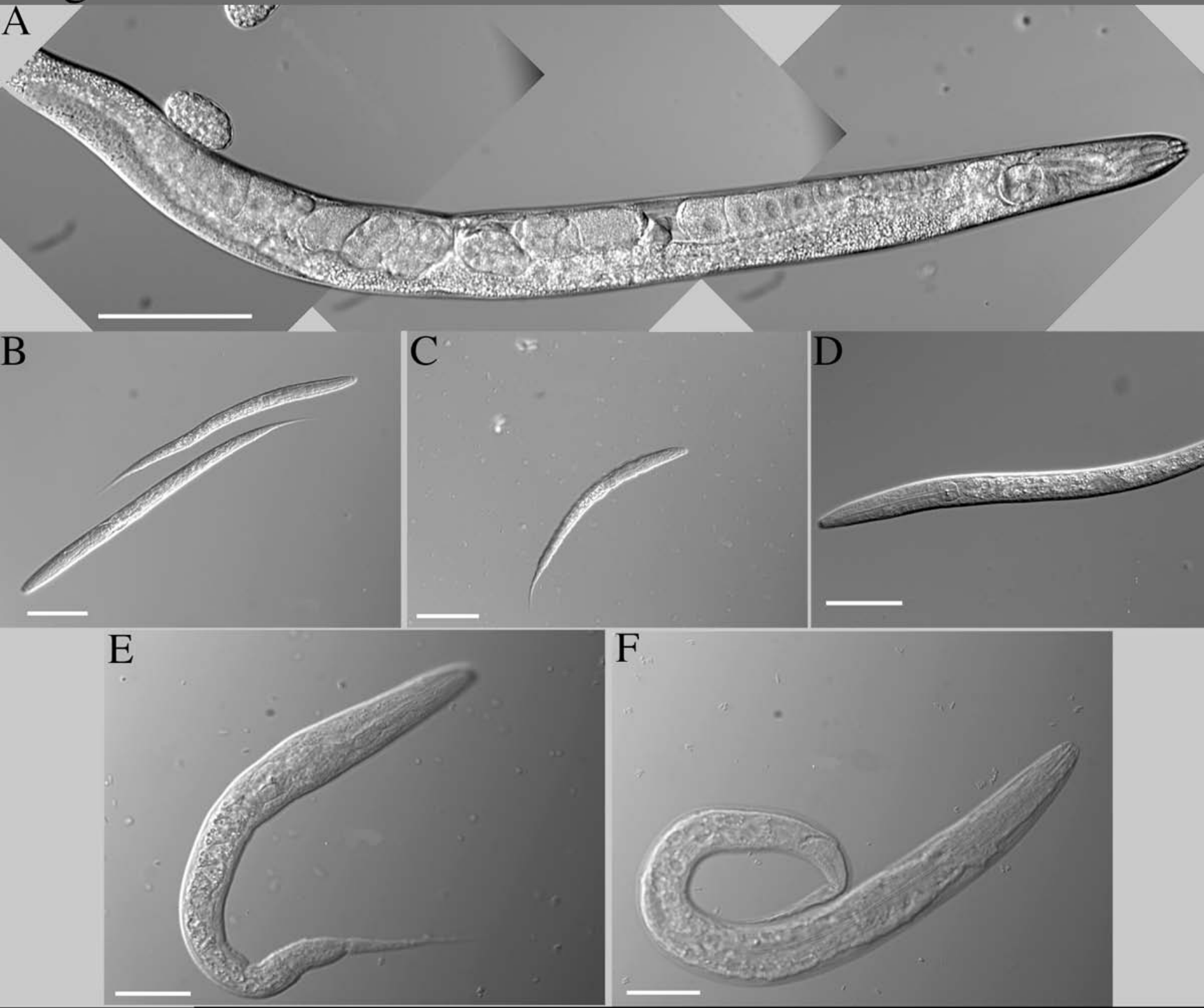

Figure 5

Figure 6

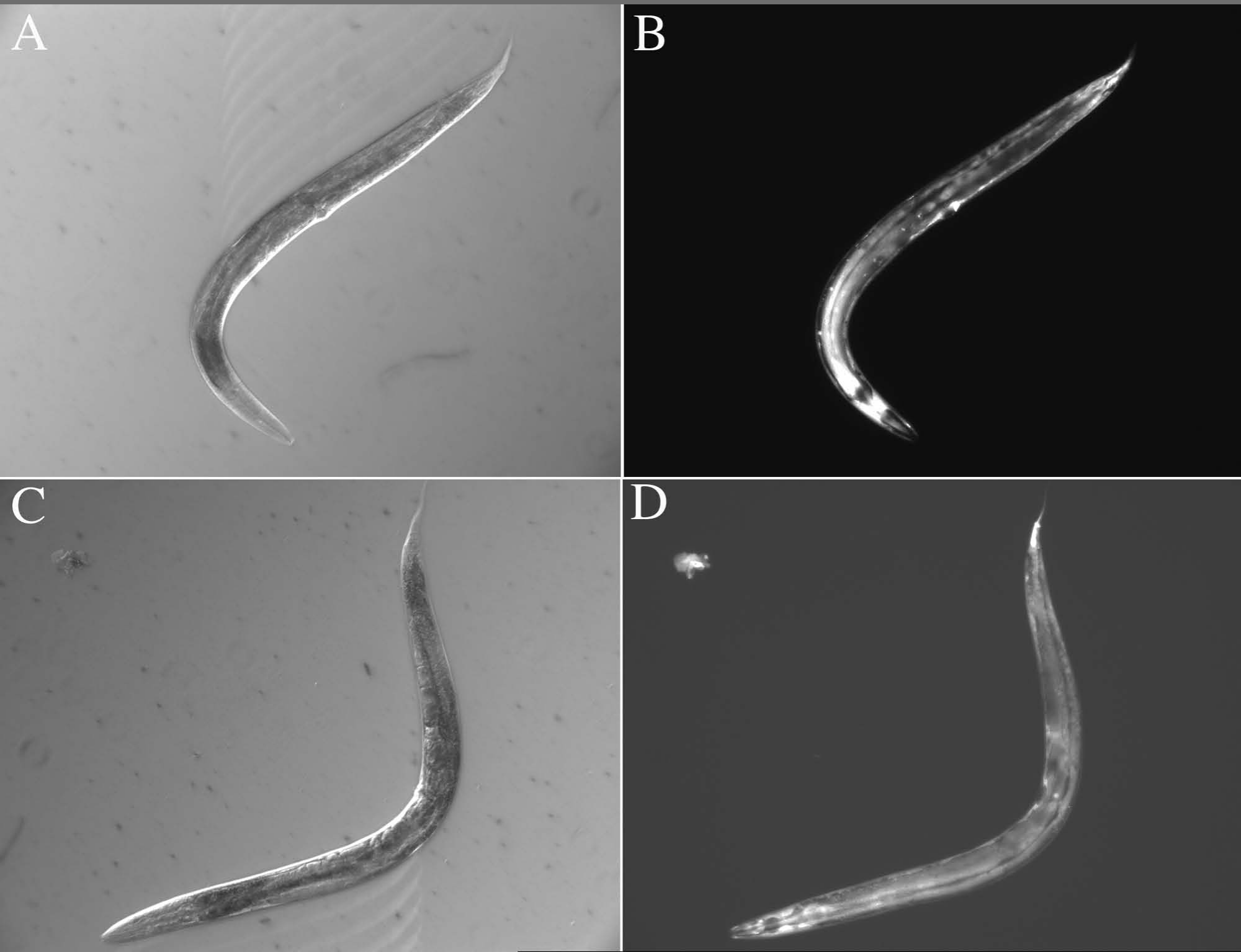

Figure 7

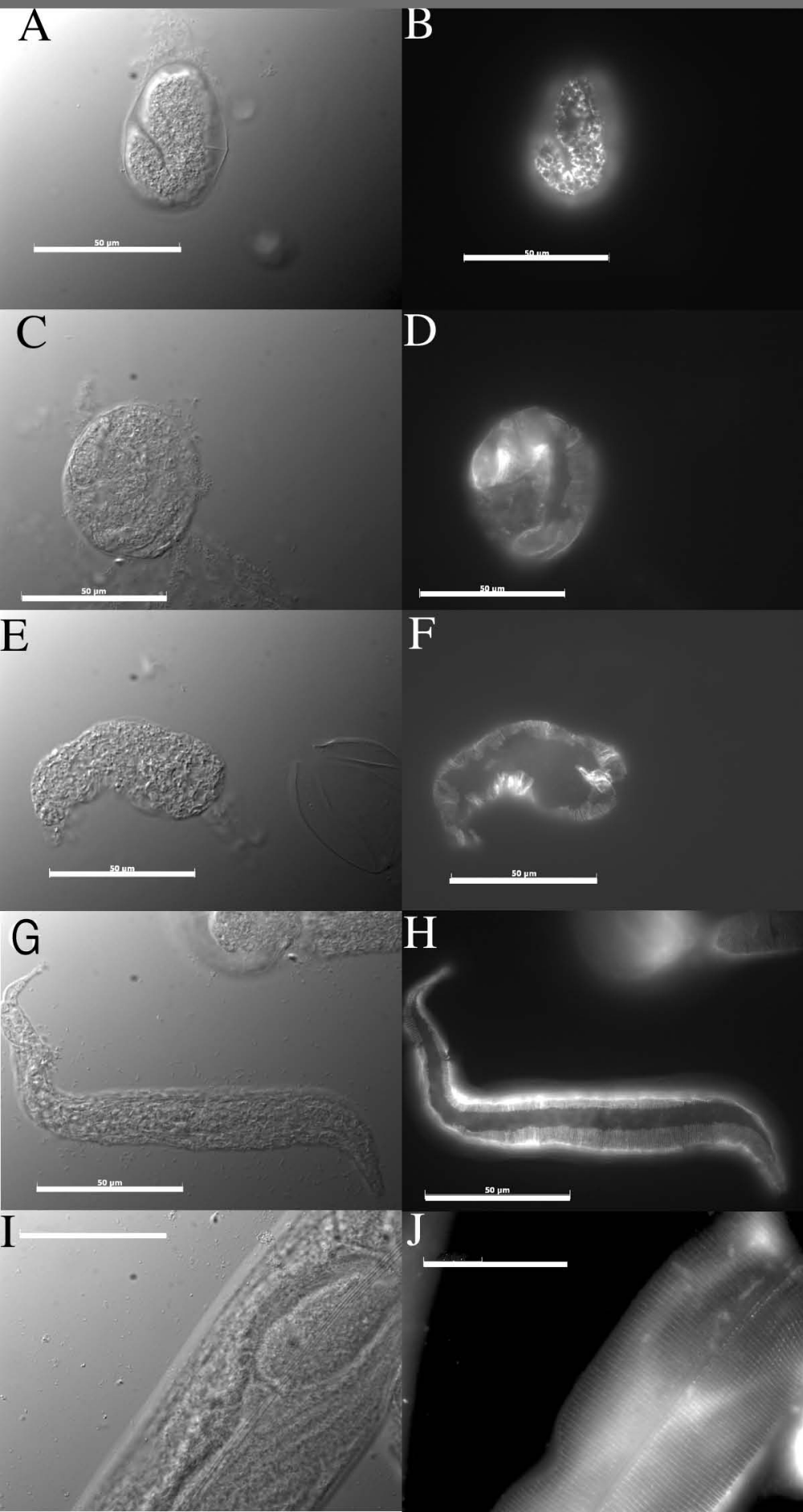

Figure 8