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The kunitz domain protein BLI-5 plays a functionally conserved role in cuticle formation in a diverse range of nematodes.

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Note: Nucleotide sequence data reported in this paper are available in the GenBank™ database under the accession numbers FJ812515 (*H. contortus bli-5*) and FJ812516 (*B. malayi bli-5*).

ABSTRACT

The cuticle of parasitic nematodes performs many critical functions and is essential for proper development and for protection from the host immune response. The biosynthesis, assembly, modification and turnover of this exoskeleton has been most extensively studied in the free-living nematode, *Caenorhabditis elegans*, where it represents a complex multi-step process involving a whole suite of enzymes. The biosynthesis of the cuticle has an additional level of complexity, as many of the enzymes also require additional proteins to aid their activation and selective inhibition. Blister-5 (BLI-5) represents a protein with a kunitz-type serine protease interacting domain and is involved in cuticle collagen biosynthesis in *C. elegans*, through its interaction with subtilisin-like processing enzymes (such as BLI-4). Mutation of the *bli-5* gene causes blistering of the collagenous adult cuticle. Homologues of BLI-5 have been identified in several parasitic species that span different nematode clades. In this study, we molecularly and biochemically characterize BLI-5 homologues from the clade V nematodes *C. elegans* and *Haemonchus contortus* and from the clade III filarial nematode *Brugia malayi*. The nematode BLI-5 orthologues possess a shared domain structure and perform similar *in vitro* and *in vivo* functions, performing important proteolytic enzyme functions. The results demonstrate that the *bli-5* genes from these diverse parasitic nematodes are able to complement a *C. elegans bli-5* mutant and thereby support the use of the *C. elegans* model system to examine gene function in the experimentally less-amenable parasitic species.

Keywords: collagen; cuticle; Caenorhabditis elegans; Haemonchus contortus; Brugia malayi.

1. Introduction

Parasitic nematodes, which cause significant debilitating infections in humans, livestock and plants, are encased in a protective barrier known as the cuticle. This exoskeleton protects the nematode from the external environment, maintains body morphology and permits motility of the nematode through opposed muscles. The cuticle is constructed from highly cross-linked small collagen-like proteins, which are modified by a variety of biosynthetic enzymes [1-3]. Monomeric collagen molecules are identified by the repeat sequence Gly-X-Y, where X and Y commonly represent proline and hydroxyproline, and three monomers combine to form a triple helical structure that in turn makes up the mature collagen molecule [1]. Over 150 collagen molecules are present in the genome of the free-living nematode *Caenorhabditis elegans* [1]. Disulphide bonding and covalent di- and tri-tyrosine cross-links are essential to stabilise the triple helical structure and to ensure the proper structure of the molecule [3, 4]. The N-termini of these proteins contain 80-150 amino acids of non-repetitive sequence, preceding the Gly-X-Y repetitive domain, which contains a signal peptide sequence for transportation of the protein to the endoplasmic reticulum, followed by a conserved subtilisin-like pro-domain cleavage site [2]. A non-repetitive region is also present at the C-terminus, following the Gly-X-Y repeat domain and this, in turn, in certain collagens, contains an astacin-like processing domain [5].

Large families of cuticle collagen genes are also present in parasitic nematodes, such as *Haemonchus contortus* [6], *Ascaris suum* [7, 8], *Brugia malayi*, *B. pahangi*, *Onchocerca volvulus*, *Dirofilaria immitis* [9] and *Ostertagia circumcincta* [10], showing great similarity in structure to those found in *C. elegans* and suggesting a common mode of biogenesis and a specific role for collagen in cuticle function throughout the nematode phylum [8, 11]. All

nematode species undergo four developmental moults from the first-stage larva to the mature adult, with the cuticle being re-synthesised prior to each moult. The correct assembly of the nematode cuticle at each stage consists of several critical steps, each directed by specific enzymes [1, 2]. Following trimerisation, procollagen processing at the N- and C-termini by enzymes such as BLI-4 [12] and DPY-31 [13] permits the assembly and crosslinking of mature collagens into the matrix. It is this procollagen processing step that is the focus of this study. The C-terminal and N-terminal non-Gly-X-Y regions both encode essential pro-collagen processing sites; for example, the kex2/subtilisin-like serine endoprotease BLI-4 cleaves the N-terminal site [1] and the astacin metalloprotease DPY-31 cleaves the essential collagen SQT-3 at the C-terminal non-repeat region [5]. The mode of induction and regulation of this cleavage activity remains to be elucidated.

BLI-5 has previously been proposed to be an extracellular serine protease inhibitor due to the presence of a bovine pancreatic trypsin inhibitor domain [14]. It is a crucial regulator of moulting in *C. elegans* and has been hypothesised to inhibit collagen-processing enzymes (such as DPY-31) or to regulate subtilisin-like processing enzymes (such as BLI-4) [14]. This protein contains two important characteristic domains: a nematode-specific EB domain (Pfam PF01683) and a kunitz/ bovine pancreatic trypsin inhibitor domain (Pfam PF00014). The function of the EB domain remains elusive, although it has been found, associated with kunitz domains, in several *C. elegans* proteins. The kunitz domain has been associated with members of the serine protease inhibitor class. The *C. elegans* mutant *bli-5(e518)* contains a single EMS-induced point mutation in the EB domain, resulting in an amino acid change, at position 56, from serine to leucine. This mutation and the corresponding RNAi phenotype affects the structure of the cuticle, resulting in a

blistered cuticle that becomes apparent in the adult stage, indicating that the encoded protein has an essential function in the direct regulation of cuticle synthesis and the moulting process [14].

The parasitic clade V nematode *Haemonchus contortus* is an economically important parasite of grazing livestock and represents an ideal system in which to verify and validate potential nematode-specific drug targets identified in the *C. elegans* model system. The clade III human infective filarial parasite *Brugia malayi* is the focus of a well-advanced sequencing project [15], making it a good complimentary system in which to examine conservation of gene function between evolutionary diverse nematode species. This study describes the detailed molecular and biochemical characterization of BLI-5 in both *H. contortus* and *B. malayi*.

2. Materials and Methods

2.1. Nematode strains

The wild-type Bristol N2, CB937 *(bli-4 e937)* and CB518 *(bli-5 e518)* strains of *C. elegans* were provided by the *Caenorhabditis* Genetics Center, University of Minnesota. The *H. contortus* nematodes were provided by Dr. Frank Jackson, Moredun Research Institute and the *B. malayi* nematodes were provided by Prof. Rick Maizels, University of Edinburgh.

2.2. Preparation of H. contortus and B. malayi genomic DNA, RNA and cDNA

Genomic DNA was isolated from *H. contortus* or *B. malayi* adult worms using a standard protocol involving homogenization in proteinase K, followed by repeated

phenol/chloroform extraction. Total RNA was isolated by Trizol (Invitrogen) extraction and cDNA was prepared using the AffinityScript Multiple Temperature cDNA synthesis kit (Stratagene).

2.3. Identification of the bli-5 gene from H. contortus

The *C. elegans* BLI-5 protein sequence (F45G2.5) was obtained from the Wormbase database (http://www.wormbase.org/) and a BLAST search of the Wellcome Trust Sanger institute *H. contortus* database (http://www.sanger.ac.uk/Projects/H_contortus/) was performed using the option "assembled contigs, all reads (12/11/07)". The contig 0004834 had the highest homology score, and the Genewise program (http://us.expasy.org/) was used to predict the intron-exon splice sites and the predicted coding sequence was translated (http://us.expasy.org/). The protein was aligned with the *C. elegans* protein sequence using ClustalX and BoxShade (http://www.ch.embnet.org/software/BOX_form.html) and SignalP confirmed the presence of a signal peptide. Gene Structure Draw was used to produce a scaled schematic depicting the positions of the introns and exons in the gene.

2.4. Identification of the bli-5 gene from B. malayi

A BLAST search of the *B. malayi* database (http://www.tigr.org/tdb/e2k1/bma1/) using the *C. elegans bli-5* gene identified a homologue, locus Bm1_03495. The DNA and protein sequences were analysed as described above for the *H. contortus* homologue.

2.5. Generation of H. contortus and B. malayi bli-5 rescue constructs

A 1777bp *Pst*I-*BamH*I *C. elegans bli-5* promoter construct was generated by PCR from *C. elegans* N2 genomic DNA using the primers, Ce bli-5 promF (5′-gcgctgcagctgtacctcgagacgtgggcg-3′) and Ce bli-5 promR (5′-cgcggatccggttctgaaactaaacgc-3′) and cloned into pBlueScript SK- (Stratagene). A 417bp *Xba*I-*Sac*II *C. elegans bli-5* 3′-UTR was generated by PCR from *C. elegans* N2 genomic DNA using the primers, Ce bli-5 3utrF (5′-gcgtctagaggtttttggttccacac-3′) and Ce bli-5 3′utrR (5′-cgcccgcggtgacgatgttagtttccttcac-3′), and cloned into the Ce bli-5 promoter-pBlueScript construct to create the plasmid p*bli-5* (Fig. 1B).

The genomic sequence of the *H. contortus bli-5* gene was amplified by PCR using the primers, Hc bli-5 SpeI F (5′-gcgactagtatgaagacggcattactttc-3′) and Hc bli-5 XbaI R (5′-cgctctagattaggcaaagatatttacac-3′) with *PfuUltra* polymerase to generate a product of 1622bp. This was cloned into the *Spe*I-*Xba*I digested p*bli-5* vector to create an *H. contortus bli-5* rescue construct (Fig. 1B).

The cDNA sequence of the *B. malayi bli-5* gene was isolated by PCR using the primers, Bm bli-5 F (5′-gcgggatccatgaggatatacgtaatac-3′) and Bm bli-5 R (5′-cgctctagatcagattgattgattgatg-3′) with *PfuUltra* polymerase to generate a product of 573bp. This PCR product was cloned into pCR-TOPO2.1, and a synthetic intron (5′-gtaagtttaaactattcgttactaactaactttaaacatttaaattttcag-3′) was inserted by ligation of a double-stranded oligo into a *Sna*BI blunt-ended restriction site. This product was then cloned into the *BamH*I-*Xba*I digested p*bli-5* vector to create a *B. malayi bli-5* rescue construct (Fig. 1B).

The *H. contortus* and *B. malayi bli-5* rescue constructs were each microinjected into the syncitial gonad of *bli-5(e518)* mutant nematodes at a concentration of 25μg/ml, together

with 5μg/ml of a p*dpy-7*::GFP fusion construct and 120μg/ml pBlueScript. Transformants were selected by GFP (Green Fluorescent Protein) fluorescence and four transgenic lines per rescue construct were produced. Single worm PCR was performed for three GFP positive and three GFP negative worms per line and compared to three wild type worms and three *bli-5(e518)* mutant worms, using the rescue primers, Hc bli-5 SpeI F and Hc bli-5 XbaI R (*H. contortus*) or Bm bli-5 inF (5′-gattccaatggttggatatg-3′) and Bm bli-5 R (*B. malayi*).

2.6. Over-expression of H. contortus bli-5

The *H. contortus bli-5* rescue construct was co-injected into wild type *C. elegans* worms at 100μg/ml with p*dpy-7*::GFP marker plasmid. Three transgenic lines were obtained and examined by U.V. microscopy. The *C. elegans bli-5* overexpression construct as detailed earlier [14] was co-injected at 140μg/ml with p*dpy-7*::GFP marker, producing two additional transgenic lines which were examined as described above.

2.7. RNA interference (RNAi)

RNAi feeding constructs for *bli-5* (F45G2.5) [14] and *bli-4* (K04F10.4) were cloned into the feeding vector L4440. For *bli-4*, a 292bp fragment was produced by PCR, using *C. elegans* N2 cDNA and the primers, Ce bli-4 F2 (5′-gatgaaagtataggtgcctgtg-3′) and Ce bli-4 R2 (5′-gatgaacatcattatccaggag-3′). *bli-4(e937)* worms were fed the *bli-5* RNAi bacteria, while *bli-5(e518)* worms were fed on the *bli-4* RNAi bacteria and, as a negative control, both strains were also fed bacteria containing the L4440 feeding vector alone. All worms were viewed daily and phenotypic observations were recorded.

2.8. Semi-Quantitative RT-PCR

Semi-Quantitative RT-PCR was performed using *C. elegans* cDNA samples taken at two-hourly intervals throughout the lifecycle from 2 to 40 hrs post-embryonic hatching. The cDNA template and the RT-PCR methods were performed as described previously [16] using the gene combinations of *bli-4* and *ama-1*, and *bli-5* and *ama-1*, where the control gene was *ama-1,* the constitutively expressed RNA polymerase II subunit. The primers used for these gene amplifications were Ce ama-1 F2 (5′-gtcgagtttccagaagtctac-3′) and Ce ama-1 R2 (5′-gtacggtacaaatcatccattc-3′), Ce bli-5 inF2 (5′-caatatgtgttcgtggaagatg-3′) and Ce bli-5 inR (5′-ggacaacaaactccatccaatg-3′), and Ce bli-4 F2 (5′-gatgaaagtataggtgcctgtg-3′) and Ce bli-4 R2 (5′-gatgaacatcattatccaggag-3′), with the resulting gene products being 634bp, 280bp and 292bp, respectively. The optimal cycling conditions were as follows: 94°C for 5mins; 35 cycles of 94°C for 1min, 56°C for 2mins, 72°C for 1min; and then 72°C for 5mins. The PCR products were analysed on a 1% agarose gel and the relative abundance of the *bli-5* and *bli-4* genes, compared to that of the *ama-1* gene, was determined from the gel images using ImageQuant TL software (Amersham).

2.9. Recombinant expression of C. elegans, H. contortus and B. malayi BLI-5 proteins

The constructs encoding the mature proteins (without signal peptide and prodomain) of *C. elegans*, *H. contortus* and *B. malayi* BLI-5 were isolated by PCR using the primers, Ce bli-5matF (5′-gcggcatgcgagaaatgcctgaccaatg-3′) and Ce bli-5matR (5′-cgcaagcttgaaaagatgaaagttgga-3′), Hc bli-5matF (5′-gcggcatgcgtcaaacatcaagcaaag-3′) and Hc bli-5matR (5′-cgcaagcttggcaaagatatttacac-3′), or Bm bli-5matF (5′-

gcggcatgcaaggaatgcaaaaatgatg-3′) and Bm bli-5matR (5′-cgcaagcttgattgattgattgatg-3′) with *PfuUltra* polymerase to generate products of 549bp, 537bp and 528bp, respectively. The PCR products were cloned into pCR-TOPO2.1, fully sequenced and sub-cloned into the *Sph*I-*Hind*III digested pQE30 (Qiagen) vector to create N-terminal histidine tagged protein expression constructs. The sequences of these plasmids were checked over the cloning junctions prior to transformation of *E. coli* M15 (pREP4) cells for expression of the encoded protein by an adapted autoinduction method [17]. Briefly, 10ml cultures of MDG medium containing 100μg/ml ampicillin and 25μg/ml kanamycin were inoculated overnight with these clones. This non-induced culture was used to inoculate 500ml of ZYM-5052 medium for 40-50 hrs at 25°C. The cells expressing the protein were collected by centrifugation at 5500rpm, 4°C for 20mins and re-suspended in native lysis buffer (pH 8.0) containing 10mM imidazole and 1mg/ml lysozyme. The lysed cells were sonicated and the soluble protein was collected by centrifugation at 11500rpm, 4°C for 30mins. Purification of the proteins from the soluble cell lysates was performed using Ni-NTA resin columns at 4°C under native conditions and the fractions were analysed by SDS-PAGE and western blotting using an Anti-His (G) antibody (Invitrogen).

2.10. Serine protease assay with recombinant BLI-5 from C. elegans, H. contortus and B. malayi

Serine protease assays were used to determine the inhibitory activity of recombinant CeBLI-5, HcBLI-5 and BmBLI-5, and were essentially as previously described [18]. Briefly, 0, 0.5, 1 and 2μM of each of the recombinant enzymes was incubated with either 3nM bovine pancreatic α-chymotrypsin, 1.5nM porcine pancreatic elastase, or no serine

protease and the volume was made up to a total of 100µl with TNC buffer (10mM Tris-HCl, pH 8.0/ 150mM NaCl/ 5mM $CaCl_2$). Equivalent concentrations of bovine serum albumin (BSA) were used as a negative control protein. Samples were incubated for 1hr at 25°C prior to the addition of either 200µM Suc-Ala-Ala-Pro-Phe-pNA (chymotrypsin substrate) or 250µM Suc-Ala-Ala-Pro-Ala-pNA (elastase substrate) then incubated at 25°C for a further 1hr. Absorbance was measured on a OpsysMR (Dynex) plate reader at 450nm, with each set of samples being examined in triplicate.

2.11. Microscopy and Imaging

All nematodes were transferred to 2% agarose (0.06% sodium azide) pads and viewed under Differential Interference Contrast (DIC) or fluorescence (GFP) optics on a Zeiss Axioskop2 microscope, and images were taken using an AxioCam camera and Axiovision software.

3. Results

3.1. Identification of H. contortus and B. malayi bli-5 homologues

The BLAST search of the *H. contortus* database revealed contig 0004834 to have the highest homology score with *C. elegans* BLI-5. The intron-exon splice sites were predicted from this sequence using the GeneWise program on the ExPASy proteomics website, and the coding sequence was translated through the ExPASy site and then aligned with the *C. elegans* homologue using ClustalX and BoxShade. A BLAST search of the *B. malayi* genome database identified a homologous *bli-5* gene in the filarial nematode, *B. malayi* (locus Bm1_03495). The gene structure and comparison of the nematode *bli-5* homologues

are presented in Table 1 and Figure 1A. The full-length coding sequence for *B. malayi bli-5* is trans-spliced to the SL1 spliced leader whereas the *C. elegans* and *H. contortus* transcripts are not (data not shown).

A signal peptide cleavage site was predicted by the SignalP program to be between Ala-17 and Val-18 for *H. contortus* BLI-5, between Cys-20 and Glu-21 for *C. elegans* BLI-5 and between Ala-15 and Lys-16 for *B. malayi* BLI-5 (Fig. 2A). These BLI-5 proteins range from 19-20kDa and have isoelectric points that range from 5.26 to 5.98 (Table 1) and share between 40-50% identity to *C. elegans* BLI-5 (Fig. 2A). A BLI-5 homologue is also present in the parasitic nematode *Ostertagia ostertagi* (Accession number: BQ457535.1) that shares 49.3% identity to the *C. elegans* protein. All nematode proteins contain the conserved nematode-specific EB domain (Pfam PF01683), SREWVCL, and the kunitz-type serine protease inhibitor domain (Pfam PF00014) (Fig. 2). The *C. elegans* EB domain shares 100% identity with *O. ostertagi* and *H. contortus*, but only 57% identity to *B. malayi*. The C-terminal kunitz-type serine protease inhibitor domains show highest levels of identity between *C. elegans*, *H. contortus* and *O. ostertagi* (67%) (Fig. 2A). This consecutive arrangement of EB and kunitz-type domains is not found outside the phylum nematoda.

3.2. Complementation of the bli-5(e518) mutant worms with the H. contortus and B. malayi orthologues

The *C. elegans bli-5(e518)* mutant was transformed via microinjection with a construct expressing the *H. contortus bli-5* gene along with a *C. elegans* promoter GFP marker. Four transgenic lines were obtained and adults were examined for the restoration of the wild

type appearance characteristic of the N2 strain (Fig. 3A) from the mutant blistered cuticle appearance (Fig. 3B). The *H. contortus bli-5* genomic construct successfully rescued the *bli-5* mutant worms, restoring their normal body morphology (Figs. 3C and 3D). Single worm PCR indicated that only the GFP positive worms for each transgenic line amplified the correct transgenic product of 1622bp (Fig. 3G), confirming that the transgenic worms contained the *H. contortus bli-5* rescue construct.

A *B. malayi bli-5* genomic rescue construct was prepared as for the *H. contortus* construct. As a consequence of mis-processing of this construct in *C. elegans*, no rescued lines were obtained (data not shown). An expression construct using a cDNA that incorporated a synthetic intron was therefore constructed to overcome this problem. However, from the population of cloned and sequenced *B. malayi* cDNAs, several were found to utilize an alternative splice site, between exons one and two, to that of the original *B. malayi* sequence (278/279bp instead of 286/287bp; Fig. 1C), thereby disrupting the translational context of *B. malayi bli-5*. A correctly spliced *B. malayi* cDNA rescue construct was therefore selected and injected into the *bli-5(e518)* mutant. This correctly spliced cDNA construct successfully rescued the corresponding *C. elegans* mutant strain (Figs. 3E and 3F) and single worm PCR confirmed that only the GFP positive worms for each transgenic line amplified the corresponding rescue construct (Fig. 3H).

3.3. Over-expression of H. contortus bli-5 produces larval body morphology defects

Transformation of wild type *C. elegans* with a high concentration of the endogenous *bli-5* rescue construct was previously shown to induce severe overexpression phenotypes, including a range of body morphology defects [14], a result that was supported by the

generation of two further over-expression lines in this study (data not shown). A similar overexpression phenomenon was observed when the *H. contortus bli-5* rescue construct was injected into wild-type *C. elegans* worms at a concentration four-fold greater than that used for transgenic rescue (Fig. 4). The over-expression phenotype was characterized by a range of L1 body morphology defects, including dumpy and moult defects (Fig. 4). This result suggests that BLI-5 plays a functionally conserved role in the normal development of both nematode species. Phenotypes were limited to the L1 stage and these went on to develop to morphologically normal adults (results not shown).

3.4. RNA interference of bli-5 (F45G2.5) and bli-4 (K04F10.4)

In all the RNAi experiments performed, no embryonic death was noted and the levels of morphological defects are summarised in Table 2. As expected, the *bli-5* RNAi feeding experiment produced an adult specific blister phenotype in 43% of the wild type (N2) (Fig. 5B) but at the higher rate of 73% when fed to *bli-4(e937)* mutants (Fig. 5G), compared to the controls comprising N2s fed the L4440 vector alone (Fig. 5A). A similar blister phenotype was present, but at the lower penetrance of 14%, in *bli-5(e518)* worms fed the control L4440 vector (Fig. 5C) and at 19% in *bli-4(e937)* worms fed the L4440 control vector (Fig. 5F). It is significant to note however that *bli-4* RNAi caused a highly penetrant, 97.5%, larval arrest phenotype when fed to either N2 (Fig. 5E) or *bli-5(e518)* mutant worms, 73% (Fig. 5D). The larval arrest phenotype occurred at the L1 stage with the L2 failing to escape from the L1 cuticle. The *bli-4* RNAi caused a more severe phenotype than the *e937* mutant allele of *bli-4*. The *bli-4* gene encodes nine alternatively-spliced isoforms and only five of these are affected by the *e937* mutation allele whereas all nine are affected

by RNAi. The fact that *bli-5* RNAi phenocopies the *bli-5(e518)* mutant allele confirms this to be a null, loss-of-function mutant allele of this gene. The more severe *bli-4* RNAi phenotype described in this study also suggests that BLI-4 plays a more important role in the correct formation of the cuticle than that of BLI-5. The RNAi experiments also indicate a mild synergistic effect between the two gene products, since there is a higher penetrance of the blister phenotype associated with *bli-5* RNAi in the *bli-4(937)* mutant background compared to the wild type strain and, more importantly, the number and severity of the blisters present are greater (Fig. 5G compared to Fig. 5B).

3.5. Expression of bli-4 and bli-5 throughout the post-embryonic C. elegans lifecycle

In an attempt to gauge the relative abundance of *bli-4* and *bli-5*, and to establish if they had overlapping expression levels, a semi-quantitative RT-PCR approach was applied using staged cDNA samples that represented two hour developmental increments from hatching into adulthood. Transcripts of 634bp, 280bp and 292bp were amplified for *ama-1*, *bli-5* and *bli-4*, respectively, with *ama-1* representing the constitutively expressed control gene. The *bli-4* transcript was relatively constitutively expressed, being detected in all *C. elegans* lifecycle stages, but displayed peaks of abundant expression at each larval transition, most significantly at the L2-L3, L3-L4 and L4- adult moults (Fig. 6A). *bli-5* was found to be less abundantly expressed than *bli-4* but displayed peaks of expression at the L2-L3 moult at 18 hrs, then another peak prior to the L3-L4 moult, from 22 to 24 hrs (Fig. 6B).

3.6. Expression of C. elegans, H. contortus and B. malayi active BLI-5 recombinant proteins

The genes encoding the mature BLI-5 proteins from each of the three nematode species were cloned and inserted into the pQE30 expression vector to allow expression of histidine-tagged proteins. The recombinant BLI-5 proteins were expressed by an autoinduction method, purified on Ni-NTA columns and an anti-His antibody was used to detect a molecule of approximately 25kDa from each species following Western transfer (data not shown).

The recombinant proteins were then assessed for their potential inhibitory activities in a serine protease assay with five separate serine proteases. This assay, however, demonstrated that the recombinant BLI-5 proteins did not in fact inhibit the serine protease activity, but instead they enhanced this activity (data not shown). To confirm this finding, a range of concentrations of each of the BLI-5 nematode proteins were incubated with two distinct classes of serine proteases, namely porcine pancreatic elastase and bovine pancreatic α-chymotrypsin. There was a positive correlation between the nematode BLI-5 concentration and absorbance at 450nm, which corresponds to substrate cleavage (Fig. 7A). Additionally, when the BLI-5 proteins are incubated with substrate in the absence of the serine protease, equivalent substrate cleavage was also shown to occur (Fig. 7B). Therefore, BLI-5 appears to be an active enhancer of serine protease activity, or indeed a proteolytic enzyme and not an inhibitor, as previously hypothesised, and this role is conserved between the recombinant proteins from the three diverse nematode species.

4. Discussion

In this paper, we describe the identification and biochemical characterization of the nematode-specific kunitz domain protein, BLI-5, from the parasitic nematodes *H. contortus* and *B. malayi* and from the free-living nematode *C. elegans*. BLI-5 from *H. contortus, O. ostertagi* and *C. elegans* share the highest identity, a fact consistent with them belonging to the same phylogenetic clade [19].

Despite the difference in identity of the EB domain between the nematode BLI-5 homologues, there is an important functionally conserved serine (position 56) found in all four nematode species. This position corresponds to the point mutation in the *C. elegans bli-5(e518)* mutant allele that results in a leucine substitution [14]. The functional relevance of this BLI-5 conservation was confirmed through the rescue of the *C. elegans* mutant with either the *H. contortus* or *B. malayi* gene, as evidenced by the restoration of normal cuticle structure. It is highly significant that this functional conservation extends to the distantly related clade III nematode *B. malayi.* There are numerous published examples of successful interspecies complementation between *H. contortus* and *C. elegans* [20, 21] but, to our knowledge, this represents the first published example of a *B. malayi* gene rescuing a *C. elegans* mutant, and this strengthens the proposition that BLI-5 plays a critical nematode-specific role in cuticle biogenesis.

The *bli-5* mutant rescue experiments performed with a *B. malayi bli-5* rescue construct also revealed that the *B. malayi* gene was spliced at two alternative intron-exon sites between the first intron and second exon, only one of which produced a translationally in-frame message that permitted rescue of the mutant body form (Fig. 1C). This is in contrast to the single in-frame transcript produced by *H. contortus bli-5* in *C. elegans*. An additional difference between the splicing mechanism of the *B. malayi* and *C. elegans bli-5*

orthologues is that the *B. malayi* mRNA is trans-spliced to SL1 whereas the *C. elegans* mRNA is not.

The specific function of the nematode-specific EB domain remains to be established, although it has been found associated with the kunitz-type bovine pancreatic trypsin inhibitor domains in several, as yet uncharacterised, *C. elegans* proteins. It is the presence of this latter domain that has led to the proposition that these proteins may function as serine protease inhibitors. The EB domain contains eight conserved cysteine residues that are predicted to form four disulphide bridges, and the Pfam website (http://pfam.sanger.ac.uk/) describes the EB domain as being similar to the trypsin inhibitor-like cysteine-rich domain (Pfam PF01826). This latter domain contains ten cysteine residues that form five disulphide bonds, and is found in several trypsin inhibitors and extracellular proteins, but is restricted to those proteins found in the Ecdyzoa superphylum, namely the moulting nematoda and arthropoda phyla. The presence of highly conserved cysteines in the EB, and the following domains of the BLI-5 orthologues, supports the importance of these residues in the proper folding and the structural conformation of these proteins.

A wide range of diverse nematode species have been found to express proteins with trypsin inhibitor-like domains, including; *Ascaris suum* [22], *Ancylostoma caninum* [23], *A. ceylanicum* [24], *Trichuris suis* [25] and *Onchocerca volvulus* [26]. Many of these nematode proteins have also been shown to inhibit chymotrypsin, trypsin, elastase and, in the case of the hookworms, coagulation factor Xa, and have functions predicted to be important in parasite development [25, 26].

Serine protease inhibitors with specific kunitz-bovine pancreatic trypsin inhibitor domains have also been described from a diverse range of organisms, including ticks, where they have anti-coagulant and anti-inflammatory properties [27, 28]. In *Drosophila melanogaster,* they are required for larval moulting [29, 30] and, in mice they are involved in muscle differentiation [31]. In the nematodes *H. contortus* [32], *A. caninum* [33] and *A. ceylanicum* [18, 34], these kunitz-type inhibitor domains have been predicted to have an essential role in nematode development, a role also envisaged for this domain in the BLI-5 orthologues described in this study.

Mutations or RNAi depletion of *bli-5* in *C. elegans* result in a range of body morphology defects, including blistering of the adult cuticle, that support the proposition that the protein has a function in the proper formation and development of this essential exoskeleton: a premise supported by the fact that the *bli-5* transcript is expressed in the larval and adult hypodermis, the vulva and excretory cell and duct of *C. elegans* [14]. It has been proposed that BLI-5 may function by regulating one or more of the enzymes involved in collagen processing, and two potential candidates include BLI-4 and DPY-31, both of which are directly involved in the processing of cuticular collagens in *C. elegans* [14]. BLI-4 is a calcium-dependent serine endoprotease that is synthesised as a zymogen and is a member of the kex2/subtilisin-like proprotein convertase family [35]. BLI-4 is essential for the normal development of *C. elegans* in that it catalyses the cleavage of inactive precursor proteins to mature, active forms at the specific cleavage site, R-X-K/R-R [35, 36]. This enzyme plays an essential role in development and in the production and maintenance of the adult cuticle since mutant alleles that affect all alternative forms of this enzyme (*h199, h1010* and *q508*) result in late embryonic arrest, whereas the mutation that affects only a

subset of these alternative forms (*e937*) results in blistering of the adult cuticle [35, 36]. The R-X-K/R-R cleavage site is present at the N-terminal non-repetitive region in all *C. elegans* cuticle procollagens, suggesting a role for BLI-4 in general procollagen processing. It may be predicted that the convertase activity of BLI-4 would be tightly regulated throughout development. Based on domain homology, BLI-5 was hypothesised to be an inhibitor or regulator of serine-type endopeptidase activity. Both BLI-4 [37] and BLI-5 [14] are expressed in the hypodermal cells, vulva and ventral nerve cords throughout the lifecycle of *C. elegans*. From the semi-quantitative RT-PCR results in this current study, it is clear that *bli-5* is most abundantly expressed at the L2-L3 moult and the L3-L4 moult. The results of combined *bli-4* and *bli-5* RNAi do support a mild synergistic effect, as *bli-5* RNAi in the *bli-4* mutant background is both more penetrant and more severe than *bli-5* RNAi in a wild type background. This result does however raise the possibility that *bli-5* may be part of a larger redundant family since the nematodes remain viable in the absence of *bli-5,* indeed a further 57 proteins containing the kunitz motif, two of which also posses EB domains, can be found in the Wormbase database. *bli-4,* on the other hand, is essential since its removal by RNAi or mutations that affect all transcripts leads to early L1 larval arrest [11, 35]. It is interesting to note that the overexpression of *H. contortus* BLI-5 in *C. elegans* also specifically affects the L1 stage, causing body morphology defects. The specific sensitivity of this stage could relate to the fact that this represents the first collagenous cuticle, whose major role is maintenance of proper body morphology, a role prior to this stage that is dependant on actin fibres [38]. The L1-specific sensitivity to increased levels of BLI-5 could, alternatively, relate to the normally low abundance of this protein during this stage (Fig. 6B).

The results from our *in vitro* serine protease assays suggest that recombinant BLI-5 is in fact a proteolytic enzyme, as opposed to an inhibitor, of serine protease activity. Further support for this hypothesis comes from studies performed in the *D. melanogaster* basement membrane protein papilin, a protein that is essential for embryonic development of the fly. Based on its homology to kunitz-type bovine pancreatic trypsin inhibitor domains, papilin was proposed to be an inhibitor of an ADAMTS enzyme. This protein has likewise recently been shown not to possess serine, trypsin nor chymotrypsin protease inhibiting activity [39, 40]. This finding was consistent with the absence of key residues from the bovine pancreatic trypsin inhibitor motif ($Fx_{(3)}GCx_{(6)}FYx_{(5)}C$); [32, 40]. This observation may also explain the absence of serine protease inhibitory activity that we describe for the nematode BLI-5 proteins, since the identity between the kunitz domain of the BLI-5 protein from each of the nematode species and bovine pancreatic trypsin inhibitor is only approximately 20% (Fig. 2B).

The *C. elegans* cuticle collagens contain the N-terminal R-X-K/R-R cleavage motif [35], a domain shared by *H. contortus*, *A. suum* and *B. pahangi*, indicating that this mechanism of collagen maturation is conserved across the nematode families [37]. BLI-4 is likewise conserved in other nematodes with homologues found to date in *O. volvulus*, *H. contortus*, *S. stercoralis*, *A. caninum*, *T. spiralis*, *D. immitis*, *A. suum*, *A. ceylanicum* and *B. malayi* [11, 15, 37, 41, 42] (and our unpublished results). BLI-4 and BLI-5 represent nematode-specific proteins that play important developmental roles in the formation of the nematode cuticle and may therefore represent novel targets for the control of parasitic nematodes of medical and economic importance.

Acknowledgements

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Table 1. Comparisons of the genomic and coding sequences of the *bli-5* gene and of the BLI-5 mature protein between *C. elegans*, *H. contortus* and *B. malayi*

	C. elegans	*H. contortus*	*B. malayi*
Intron number	3	3	2
Exon number	4	4	3
Gene size (genomic)	1991bp	1622bp	983bp
Gene size (coding)	609bp	588bp	573bp
pI	5.98	5.42	5.26
MW (kDa)	20	20	19
no. of amino acids	182	178	175

Table 2 *bli-4* and *bli-5* RNAi in wild type and mutant *C. elegans* backgrounds

Worm strain	RNAi clone	Total no. of worms	No. affected by RNAi	% affected by RNAi
N2	L4440	548 adults	0	0%
N2	F45G2.5 (*bli-5*)	436 adults	189 (blistered)	43.3%
N2	K04F10.4 (*bli-4*)	488 L1	476 (larval arrest)	97.5%
bli-5 (e518)	L4440	103 adults	14 (blistered)	13.6%
bli-5 (e518)	K04F10.4(*bli-4*)	133 L1	97 (larval arrest)	72.9%
bli-4 (e937)	L4440	190 adults	37 (blistered)	19.5%
bli-4 (e937)	F45G2.5 (*bli-5*)	242 adults	177 (blistered)	73.1%

FIGURE LEGENDS

Fig. 1. (A) Gene structures of *C. elegans*, *H. contortus* and *B. malayi bli-5*. The introns are indicated by lines, while the exons are open boxes with Roman numerals. The translational start and stop codons are indicated by the ATG and TGA or TAA, respectively, and the conserved nematode-specific EB domain is found in exon II, and is shaded black. The kunitz-type serine protease inhibitor domain is shaded grey, and is found in exon IV for *C. elegans* and *H. contortus*, and exon III for *B. malayi*. (B) Schematic representation of *H. contortus* and *B. malayi bli-5* rescue constructs. The rescue vector p*bli-5* contains the *C. elegans bli-5* promoter and 3′-UTR sequences in pBluescript. The *H. contortus* genomic *bli-5* rescue construct was inserted into the p*bli-5* vector. The full-length *B. malayi bli-5* cDNA sequence, with a single *C. elegans* synthetic intron inserted at the 5′ end, was likewise inserted into p*bli-5*. The black boxes indicate coding regions, with the white boxes representing introns. The grey extended box on the promoter represents the six additional bases of the pBlueScript SK- vector for *Spe*I. SI, synthetic intron. (C) Alternative intron-exon splice-acceptor sites at intron 1/ exon 2 junction in *B. malayi bli-5* when expressed in *C. elegans*. Site 1 is the alternative splice site utilized by *C. elegans*, and site 2 is the in-frame splice site used by the *B. malayi* gene.

Fig. 2. (A) Alignment of nematode BLI-5 homologues. Amino acid sequences were aligned using ClustalX and BoxShade, with identical amino acids shaded black and similar amino acids shaded grey. The signal peptide domain is indicated by italics, with the ^ highlighting the conserved serine residue that is mutated to leucine in the *bli-5(e518)* mutant. The

conserved nematode-specific EB domain is in the black box with the highly conserved residues indicated by +. The Kunitz/Bovine pancreatic trypsin inhibitor domain is marked by a grey box. The 16 conserved cysteine residues are highlighted by an asterisk (*). The percentage sequence identity between *C. elegans* and the individual nematode homologues is indicated at the end of the alignment. (B) Alignment of the nematode BLI-5 kunitz domains with bovine pancreatic trypsin inhibitor (BPTI). The kunitz inhibitor motif (FxxxGCxxxxxxFYxxxxxC) is indicated below the alignment. The percentage sequence identities between BPTI and the nematode BLI-5 kunitz domains are indicated at the end of the alignment.

Fig. 3. Nematode *bli-5* orthologues complement the *C. elegans bli-5(e518)* mutant. The *H. contortus* or *B. malayi* rescue constructs were co-injected with p*dpy-7*::GFP transgenic marker and four transgenic lines were obtained for each species. These lines were examined under both DIC and GFP optics, and representative images are depicted. (A) DIC image of a wild type worm. (B) DIC image of a *bli-5(e518)* mutant worm. The blister phenotype is indicated by arrows. (C) DIC image of a *bli-5(e518)* worm rescued with the *Hc-bli-5* genomic construct. (D) GFP image of the worm in (C). (E) DIC image of a *bli-5(e518)* worm rescued with a *Bm-bli-5* cDNA construct. (F) GFP image of the worm in (E). Single worm PCR was performed for three GFP positive and three GFP negative worms per line for each rescue construct, for three wild type worms and three *bli-5(e518)* mutant worms, to confirm expression of the transgene. Only the GFP positive worms for each transgenic line amplified the correct product of 1622bp for the *Hc-bli-5* transgene (G) or 429bp for the *Bm-bli-5* transgene (H).

Fig. 4. Over-expression of *H. contortus* BLI-5 causes a body morphology defect in *C. elegans*. The *H. contortus bli-5* genomic rescue construct was co-injected with the p*dpy-7*::GFP marker plasmid into wild type *C. elegans*. Three transgenic lines were obtained and representative images are shown. (A) DIC image of L1 larvae indicating a dumpy body morphology defect (white arrow) alongside a wild type L1. (B) GFP image of the transformed worm in (A). (C) DIC image of an L1 displaying a body morphology dumpy defect. (D) GFP image of the transgenic worm in (C).

Fig. 5. Combined RNAi of *bli-4* and *bli-5*. *bli-4(e937)* worms were fed either the *bli-5* RNAi (F45G2.5) or the L4440 feeding vector bacteria, while *bli-5(e518)* worms were fed the *bli-4* RNAi (K04F10.4) or the L4440 bacteria. Representative images of adult worms are shown for each of (A-C and F-G), and representative images of larvae are shown for (D-E). (A) N2 fed with the L4440 vector only. (B) N2 fed with *bli-5* RNAi. (C) *bli-5* mutants fed with the L4440 vector only. (D) *bli-5* fed with *bli-4* RNAi. (E) N2 fed with *bli-4* RNAi. (F) *bli-4* mutants fed with the L4440 vector only. (G) *bli-4* mutants fed with *bli-5* RNAi. Scale bar = 20μm.

Fig. 6. Expression of *bli-4* and *bli-5* throughout the *C. elegans* life-cycle. Semi-quantitative RT-PCR was performed using *C. elegans* cDNA samples prepared from RNA isolated from worms synchronized at 2 hr intervals post hatching, for 40 hrs. The relative transcript levels of *bli-4* and *bli-5* to *ama-1* were determined using ImageQuant TL software. The data plotted represent the mean of three individual RT-PCR reactions ± standard error of

the mean. The resulting gene products for *ama-1*, *bli-5* and *bli-4* are 634bp, 280bp and 292bp, respectively, as indicated on the representative RT-PCR gels depicted below the graphs. (A) Expression of *bli-4*. (B) Expression of *bli-5*.

Fig. 7. Serine protease assay to determine the activity of *C. elegans*, *H. contortus* and *B. malayi* recombinant BLI-5. (A) 0, 0.5, 1 and 2μM of each of the recombinant enzymes were incubated with either 3nM bovine pancreatic α-chymotrypsin or 1.5nM porcine pancreatic elastase. Samples were run in triplicate and standard errors plotted. 0, 0.5, 1 and 2μM BSA was employed as the negative control, in place of recombinant BLI-5. BPCT = bovine pancreatic α-chymotrypsin; PPE = porcine pancreatic elastase. (B) 1μM recombinant BLI-5 was incubated in the presence and absence of 3nM bovine pancreatic α-chymotrypsin. Samples were run in triplicate and standard errors plotted.

Figure 1

Figure 1A

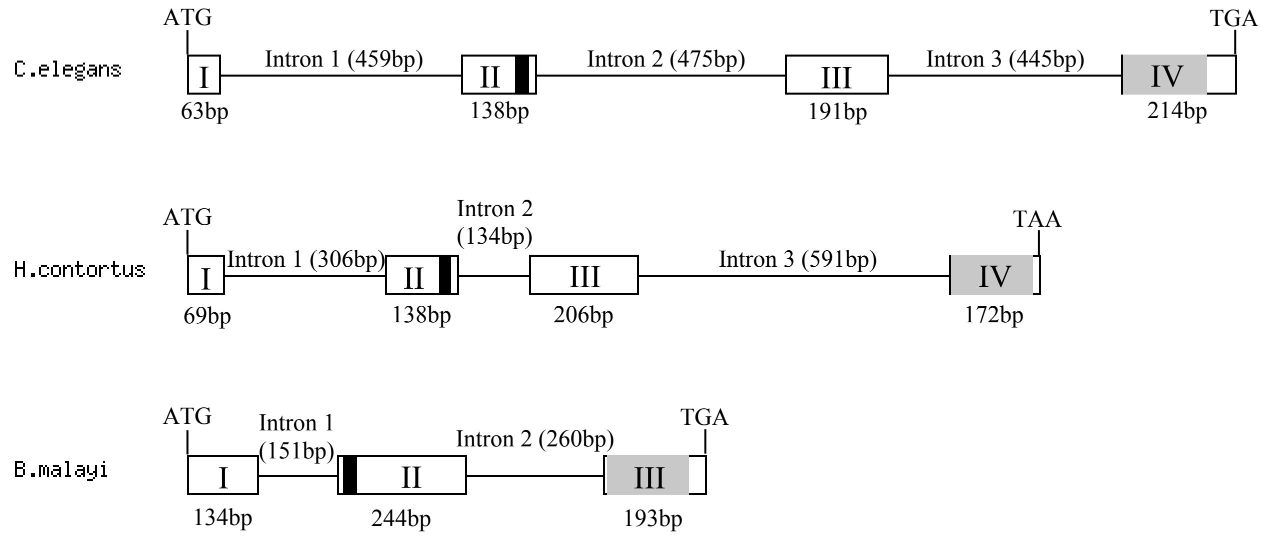

Figure 1B

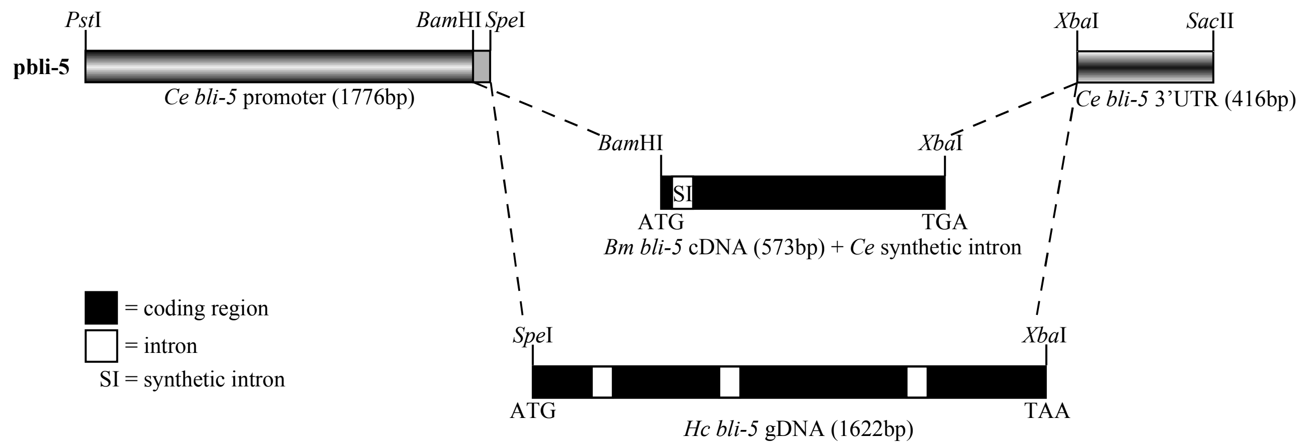

Figure 1C

270bp	intron 1	EXON 2	300bp

5′ tagatac***ag/c***aatgc**<u>ag/A</u>**AGAAAAAGTGATT 3′

site 1 site 2

Figure 2

Figure 2A

Figure 2B

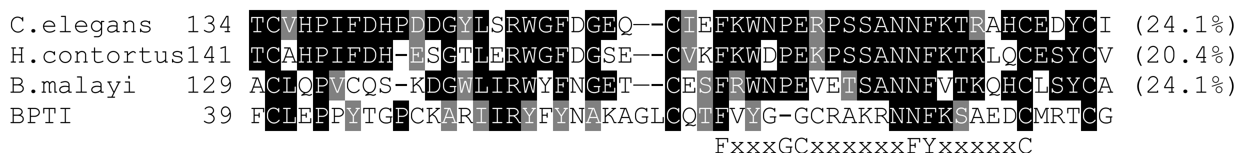

Figure 3
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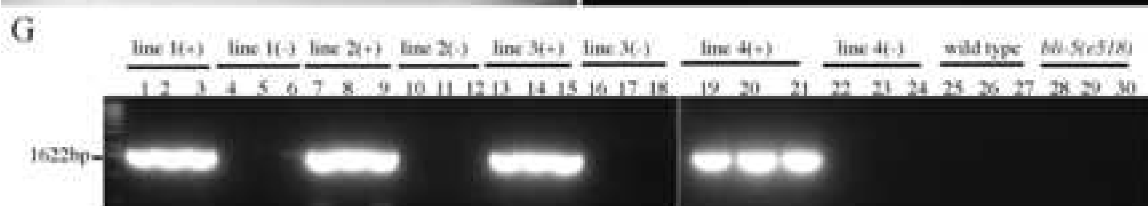
Figure 3

A B C D E F

G



H

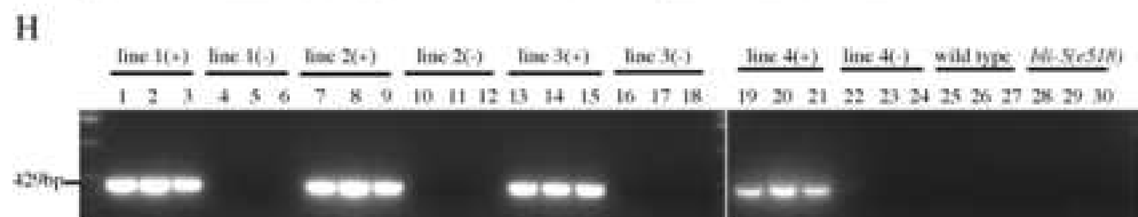


Figure 4
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Figure 4

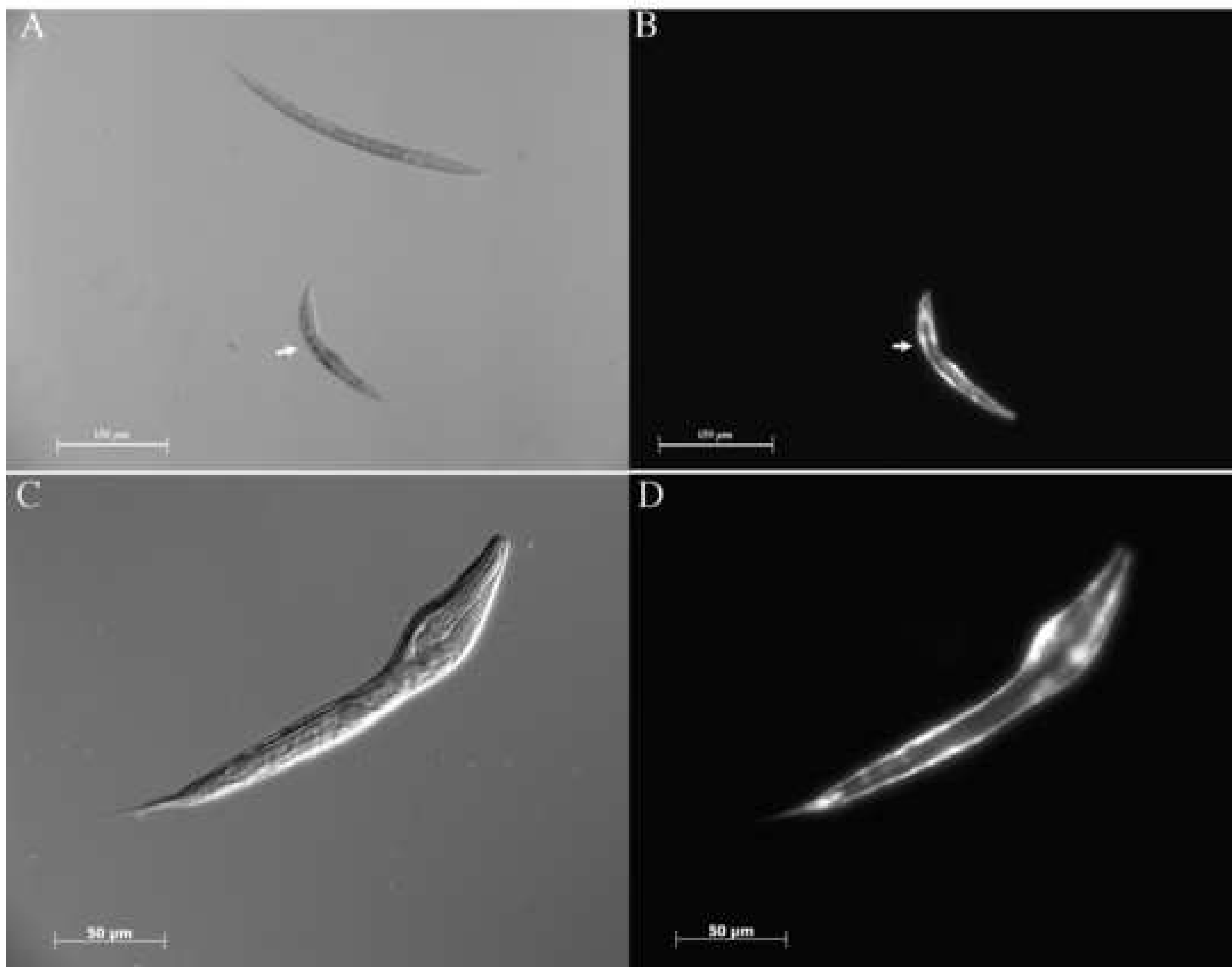

Figure 5
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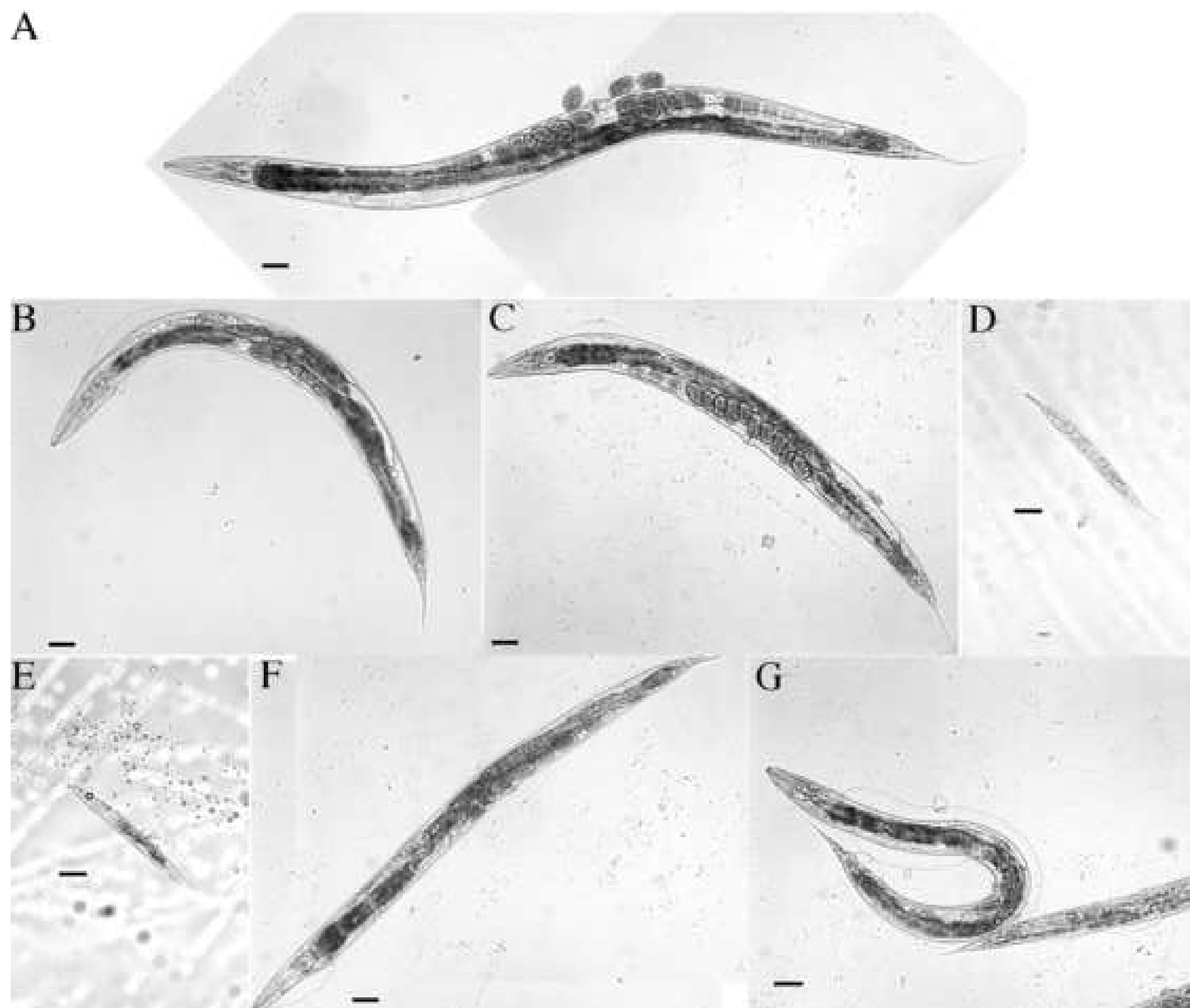

Figure 6

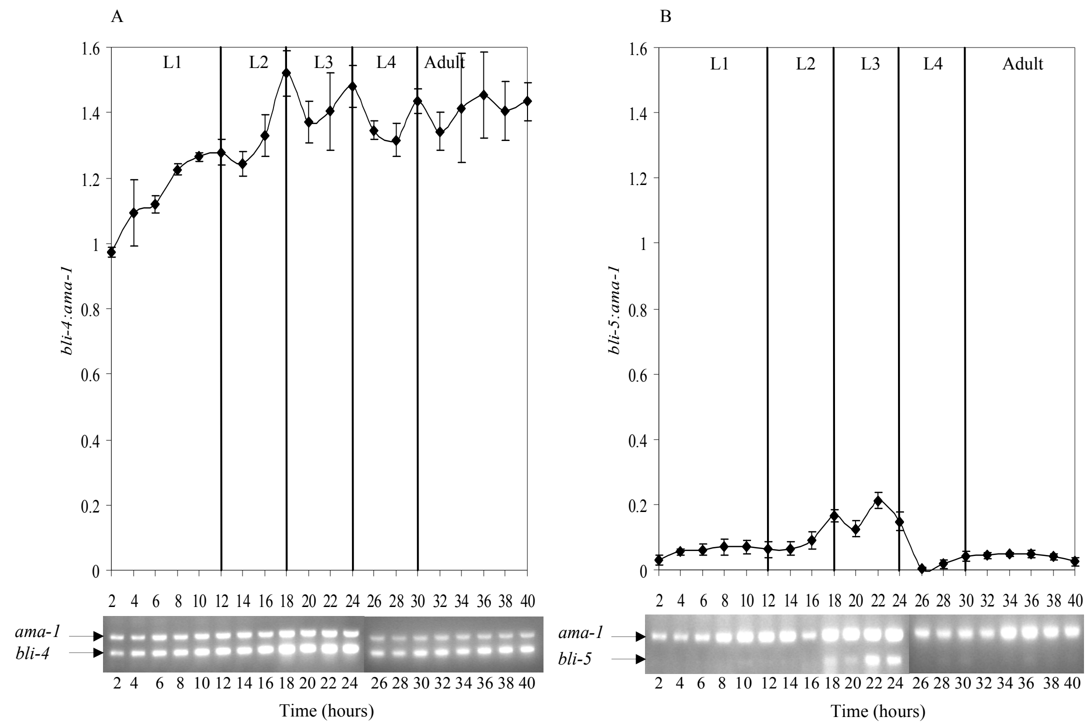

Figure 7