Caenorhabditis elegans: A comparison of nematomucin expression in surface mutants

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ABSTRACT

A class of *Caenorhabditis elegans* genes encodes amino acid sequence motifs similar to surface proteins of the parasitic nematode, *T. canis*, and therefore can serve as a model for the parasite's nematomucin expression. Putative surface protein expression in *C. elegans* surface mutants was compared to wild-type expression using anti-peptide antibodies. SDS-PAGE determined protein viability and western blotting revealed protein expression similarities. *bah-1(br1)*, *srf-2(br3)*, *bus-4(br4)*, *bah-2(br7)*, and *srf-6(yj13)* showed a 52 kDa protein band similar to the wild-type, and *bah-1(br1)* expressed an additional 36 kDa protein.

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ACKNOWLEDGEMENTS

BACKGROUND

***Caenorhabditis elegans* is Developmentally and Anatomically Similar to Other Nematodes**

As a typical nematode, the wild type *Caenorhabditis elegans* organism develops through four larval stages (L1, L2, L3, and L4) and an adult stage separated by molts during which the outer covering of the worm, the cuticle, is shed and replaced (Politz and Phillip, 1992; Riddle et al., 1997). The population exists predominantly as self-fertilizing hermaphrodites, producing sperm at the L4 stage and oocytes as an adult (Riddle et al., 1997). In conditions of crowding or food limitation, the species undergoes phenotypic morphogenesis at the second molt, becoming dauer larvae. Dauer larvae have no body openings, do not feed, and have an altered energy metabolism. This state enables the worm to live for months in starved conditions compared to the ~2 week lifespan of wild type species (Riddle et al., 1997).

The body of nematodes is constructed of two concentric tubes. The intestine comprises the inner tube, whereas the nervous system, muscle layers, and cuticle make up the outer tube as shown in Figure 1. Due to the uniformity of development and anatomy of members within the phylum Nematoda, the use of one species as a model, such as the free-living *C. elegans*, would be ideal (Politz and Phillip, 1992). With a three-day life cycle and *Escherichia coli* as a food source, thousands of *C. elegans* progeny are easily grown on a Petri dish (Riddle *et al*., 1997). These characteristics make the species favorable as a model for parasites, which have complex life cycles requiring one or more hosts, and therefore are much more difficult experimentally.
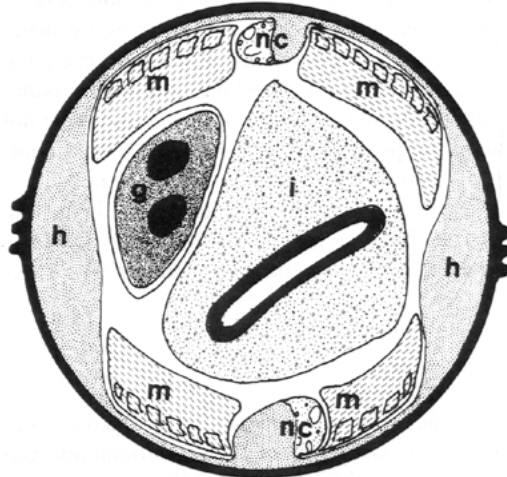
Figure 1. Posterior cross-section of an adult hermaphrodite (g- gonad, i- intestine, m- muscle, nc- nerve cord). Reproduced from Edwards and Wood 1983.

Cuticle Composition and Synthesis

The body of *C. elegans* is enclosed in an extracellular matrix termed the cuticle. In the adult stage of the nematode life, six layers of the cuticle are present: the epicuticle, external and internal cortical, medial, a honeycomb fibrous layer, and basal layers (Kramer, 1997; Peixoto et al., 1997). Synthesized by the hypodermal cells, a new cuticle is produced at four periods called molts during postembryonic development such that, with each new molt, the new cuticle is synthesized below the previous (Page & Johnstone, 2007). Expression of more than 170 cuticle collagen genes also varies with each developmental stage (Peixoto et al., 1997; Page & Johnstone, 2007). Only the adult, dauer, and L1 stage cuticles are distinct from one another, whereas the L2-L4 cuticles have similar fibrous layers (Kramer, 1997).

Although the cuticle is primarily collagen, it contains other proteins, such as insoluble cuticlin and surface glycoproteins (Peixoto et al. 1997). Cuticular collagens encoded by different *C. elegans* collagen genes are similar in that they have an N-terminal non Gly-X-Y amino domain, a central Gly-X-Y domain, and a C-terminal non

Gly-X-Y domain which is variable in length (see Figure 2). X is commonly proline and Y is commonly hydroxyproline (Kramer 1997; Page & Johnstone, 2007).
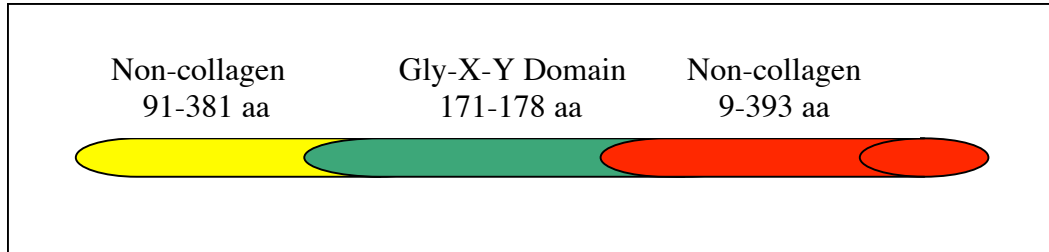

Figure 2. Domains of a cuticular collagen. Adapted from Page & Johnstone, 2007.

Above the epicuticle of nematodes is the negatively charged surface coat, which contains mucin-like glycoproteins and is 5-20nm thick (Blaxter and Bird, 1997). Mucins are O-glycosylated glycoproteins with many serine and threonine residues in the polypeptide moiety (Varki et al., 1999). They may be cell surface associated or secreted. In *Toxocara canis*, the surface coat has been shown to contain O-linked glycans and a mucin-like protein with a repeated seven amino acid sequence rich in serine (Blaxter and Bird, 1997). The N-linked and O-linked glycans of the *C. elegans* surface coat may even take part in adhesion of bacterial pathogens (Page & Johnstone, 2007).

Nematodes have the ability to alter the antigenic composition of their surface coat. This process, termed surface antigen switching, may occur by molting of the cuticle at new developmental stages or in response to environmental cues (Phillip *et al*., 1980; Grenache *et al*., 1996). During *Trichinella spiralis* development, each post-embryonic stage exhibits different surface antigens. Stage-specific immune responses directed against the infective and gut-resident stages allow later stage nematodes to avoid attack, but when reinfected by *T. spiralis*, rats are able to combat infections using previously produced antibodies (Bell *et al*., 1979; Wakelin and Denham, 1983; Appleton and McGregor, 1985). Surface antigen changes may even occur within a developmental stage.

Upon entering a new host or host tissue, the nematode may alter its surface coat composition and properties (Carlow *et al.*, 1987; Proudfoot *et al.*, 1993a,b; Modha *et al*, 1999). It is possible that *C. elegans* may discard contents of its surface coat into its surrounding environment, similar to the process observed for *T. canis* (Blaxter, *et al.*, 1992; Politz and Phillip, 1992; Politz, personal communication). Surface switching in *C. elegans* can be triggered by means of environmental cues detected by the nematodes' chemosensory system (Grenache *et al.*, 1996; Olsen *et al.*, 2006). Surface coat antigens in *C. elegans* may therefore be studied to understand the mechanism of control and expression of surface proteins, given the similarities in anatomy and development of the species of the phylum Nematoda.

The Cuticle Induces an Immune Response

The nematode surface may be responsible for the provocation of host immune responses. Nematode surface antigens have been shown to evoke a type 2 immune response in mammalian systems in which hypothesized Pattern Recognition Receptors induced IL-4 production (Tawill et al, 2004). Mice exposed to *C. elegans* antigens produced a Th2 response similar to that elicited by antigens of *Brugia malayi* and other parasitic nematodes. Carbohydrates bound to these antigens may be responsible for this type of immune response (Tawill et al., 2004). In general, extracellular peptide antigens may potentially elicit the production of Th2 cells from naïve CD4 T cells (Janeway et al., 2005). CD4 cells may differentiate into Th1 or Th2 cells and the factors determining differentiation are not completely known (Janeway *et al.*, 2005). In hosts infected by *Mycobacterium leprae*, when macrophages are activated by Th1 cells, little bacteria remains and patients typically survive. If CD4 cells differentiate into Th2 cells, however,

a humoral response occurs, which is often fatal for a patient (Janeway *et al*., 2005). Some parasitic worms also induce IL-10 production which causes anti-inflammatory cytokine production (van der Kleij *et al*., 2002).

Toxocara canis and *Caenorhabditis elegans*

Toxocara canis (*T. canis*) is a parasitic canid nematode whose transmission occurs via ingestion of the species' eggs (Center for Disease Control, 2007). Like the dauer stage of *C. elegans*, *T. canis* infective larvae are developmentally arrested and may live for many years in mammalian species other than the definitive canid host. More notably, the larvae discharge large quantities of *Toxocara* excretory/secretory (TES) antigens, which are glycoproteins found in the cuticle and surface coat (Gems and Maizels, 1996; Maizels et al., 2000). One of the most abundant antigens belongs to the TES-120 family of heavily O-glycosylated glycoproteins. cDNAs encoding Tc-muc-1, 2, 3, and 4 have been shown to encode TES-120. With the serine/threonine domain of the encoded proteins existing as about 72.1% serine/threonine at the N-terminus, this site serves as a location for O-glycosylation to occur (Gems and Maizels, 1996). Another distinct domain in TES-120 is characterized by a six-cysteine (SXC) motif which consists of two 36 amino acid sequences each with six cysteines in fixed positions (Gems and Maizels, 1996). Based on modeling studies (see Figure 3) these six cysteine residues are believed to form intramolecular disulfide bonds (Loukas et al., 2000).

When comparing the cDNAs of *T. canis* and *C. elegans*, the SXC domain has also been found to exist in at least 75 *C. elegans* genes comprised of about 300 SXC domains (Maizels et al., 2000; Emery, 2002). This domain has also been observed in other nematode species such as *Trichuris muris* and *Necatur americanus*, giving rise to

its similar name, NC6, or [Nematode (Cys)$_6$] (Blaxter, 1998; Daub *et al.*, 2000; Gems and

Maizels, 1996; Maizels *et al.*, 2000). In addition to Tc-muc-1, three other mRNAs for

apomucins have been found: Tc-muc-2-4, where Tc-muc-2 and Tc-muc-3 are secreted

mucins (Maizels et al., 2000; Loukas et al., 2000). The information available on *C.*

elegans and *T. canis* SXC sequences was used to design peptides for obtaining anti-
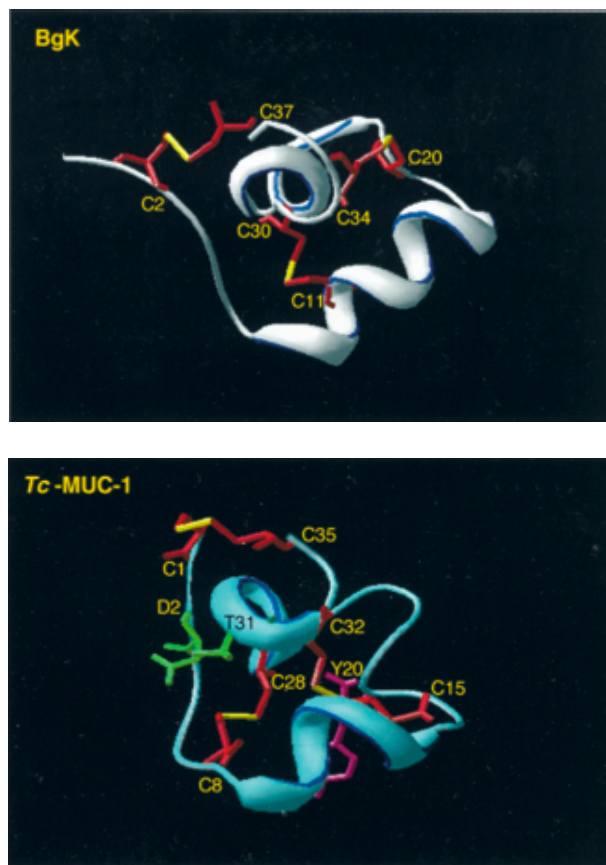
nematomucin antibody probes.

Figure 3. 3D structures of SXC domains. (Top) BgK toxin of the sea anemone *Bunodosoma granulifera* from which the Tc-MUC-1 SXC domain (bottom) was modeled. Disulfide bonds are shown in red and conserved residues of *T. canis* SXC domains are shown in green. Reproduced from Loukas et al. 2000.

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Alignment of C. elegans gene F41G3.10 exons
with T. canis Tc-MUC SXC sequences
                            *  ** *     *  ***    *****  **  *****  **********
Exon 1          MQQITLVVLAVLCSYSWGCVDLTNPRTGTSDCARLASYCTNSVYLSLMRQQCPRTCGYC
Exon 2          SSSSTATSVSSTTSSSSTCVDLTNPRTGTSDCTRLASYCTNSVYIPLMRTQCPRTCGFC
Exon 3          MQTTVLFIMAALCTVSWGCVDLTNPSNGVSDCTRLASYCTNSVYLTLMKTQCPKTCGFC
Exon 4             SSSSTSSTTSSSSTCVDLTNASTGVSDCTRMASYCTNSIYLTLMKQQCPKTCGYC
Exon 5            TTTVATTTATTATTCVDLTNPSTGVSDCPGMASYCANSAYLTLMKQQCPKTCGYC
Exon 6             TSSSATTTSSSSTCIDLTNASTGVSDCPGMASYCTNSAYLTLMKQQCPKTCGYC
Exon 7              SSSSSSSSSSCADLVNSSIGVSNCASMASYCTNSVYLSLMKTQCPKTCGYC
Exon 8            STTTRTSVTSGTCVDLTNPSTGTSDCTRMASYCFNSFYLTLMRQQCPRTCGYC
Exon 9            TGTATTTRTSTTCVDLTNPTTGVSDCTRMASYCNNSAYITLMRQQCPRTCGYC
Exon 10         SGSGVVTTTRTSTTCADLVNPNTGTSDCTAMRSYCNNSAYATLMRQQCPRTCGYC
                            *  *  *        **      *                *    *  *
Tc-MUC-1 SXC 105-140        CIDTAN------DCQLFTPLCFVQPYSRAIQGRCRRTCNIC
Tc-MUC-1 SXC 141-176        CQDSAN------DCANPVSVCLNPTYQPVLRSRCPLTCGFC
Tc-MUC-2 SXC 112-147        CIDTAN------DCQLFMPLCFVQPYSRAIQGRCRRTCNIC
Tc-MUC-2 SXC 148-172        CRDDAN------DCARLVTFCGNPMYQPVLRTRCTLTCGFC
Tc-MUC-3 SXC  34-69         CVDSAS------DCQQHTSLCFMQPYSRSMQSRCQRTCNIC
Tc-MUC-3 SXC  70-94         CRDDAN------DCARLVTFCGNPMYQPVLRTRCTLTCGFC
Tc-MUC-3 SXC 198-233        CSDAAM------DCQRYAGMCFTQPYSRAIQGRCRRTCNIC
Tc-MUC-3 SXC 234-258        CHDSAN------NCGSLISYCDDPTLQSVLRSKCPLSCGFC
Tc-MUC-4 SXC 121-156        CVDNAN------DCQVFMQLCFVQPYSRAIQGRCRRTCNIC
Tc-MUC-4 SXC 157-191        CQDTAN------DCANYVSVCLNPTYQPVLRTRCPLTCGLC
Invariant or nearly invariant residues are indicated by an asterisk.
Ser/Thr-rich sequences are shown in green.  T. canis cDNA sequences
also contain ser/thr-rich coding sequences, but these are separated
from the SXC sequences, unlike the F41G3.10 arrangement.
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Figure 4. Alignment of *T. canis* mucin sequences and F41G3.10 of *C. elegans*.

Emery (2002) aligned the amino acid sequences of the MUC-1 to -4 SXC domains in *T. canis* with the exons of F41G3.10 of *C. elegans* (see Figure 4). F41G3.10 was of interest because it maps genetically near *srf-6(yj13)*. When designing anti-peptide antibodies, the C1–C2 loop sequences were chosen since they are highly variable and, based on the 3D model shown in Figure 3, should be most accessible to antibody binding (Emery, 2002; Politz, personal communication).

Other *C. elegans* protein sequences with SXC domains were grouped based on the distances between the cysteines of the SXC motif, the location of this domain in the gene, and whether the SXC domain was "whole" or "split" (Emery, 2002). From this, five

families of proteins were created. Synthetic peptides were made for each family and contained a peptide sequence unique to that family, as shown in Figure 5.

Peptides were conjugated separately to keyhole limpet hemocyanin and anti-peptide antibodies were then made by injection of these conjugates into four rabbits. Two rabbits were injected with the peptide for the F41G3.10 family, and the others were injected with peptides representing all five of the families. Pre-immune serum and antiserum were collected. For this project, only the single peptide antibody was used.

SXC Peptide SXC Family
1. F41G3.10-5: NH2-VDLTNPSTGVSD-CONH2 3
2. T05B4.12-3: NH2-TSYAADSSTS-CONH2 5
3. F35E8.10-1: NH2-PNVAYPRLN-CONH2 2
4. T05B4.10-2: NH2-GGGGVDAVTN-CONH2 4
5. Y39G8B.g-1: NH2-GGGIDDPDVD-CONH2 2

Figure 5. Synthetic peptides representing the amino acid regions unique to the five SXC families (Politz, personal communication).

ELISA assays were performed to confirm the presence of antibodies specific for the target peptides. Antiserum and pre-immune serum were incubated with the F41G3.10 peptide and antibody binding was detected using horse-radish-peroxidase (HRP) bound secondary antibodies. Antiserum was shown to contain antibodies specific for F41G3.10 whereas pre-immune serum lacked presence of antibodies (see Figure 6).
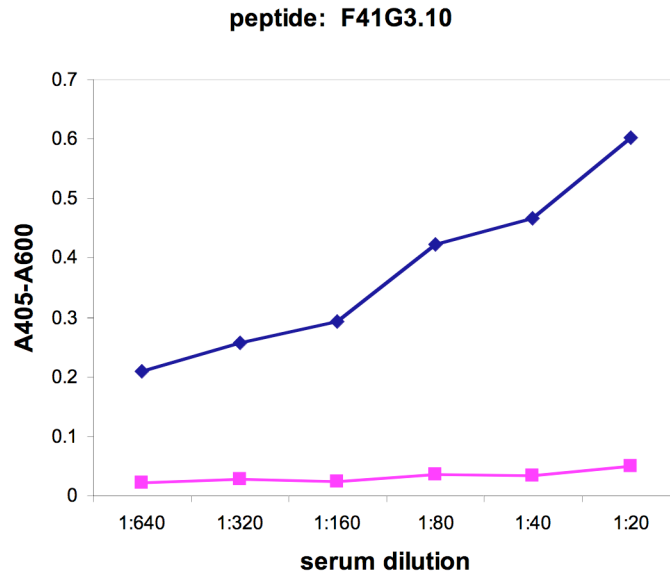
peptide: F41G3.10

Figure 6. ELISA of the F41G3.10-5 peptide incubated with pre-immune and antiserum. Results of pre-immune serum are shown in pink, antiserum is shown in blue.

Previous projects have examined the expression of cuticle proteins in mixed developmental stage protein fractions of wild-type N2 worms. Mutetwa (2007) showed, using the single-peptide antiserum, that a 52kDa protein is expressed in cuticle and total protein fractions whereas little to no expression was shown in cellular protein fractions. This is observed in Figure 7. Figure 8 also determined the preimmune serum to lack presence of any anti- F41G3.10 antibody.
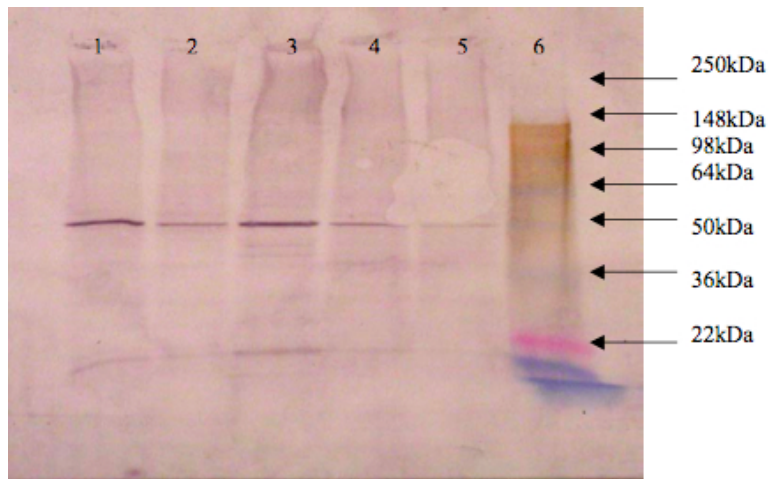
Figure 7. Mixed developmental stage N2 protein fractions probed with single-peptide antiserum. (From left) 02/11/04 cuticle protein; 02/11/04 total protein; 08/13/04 cuticle protein; 08/13/04 total protein; 08/13/04 cellular protein; Invitrogen SeeBlue® marker. From Mutetwa 2007.
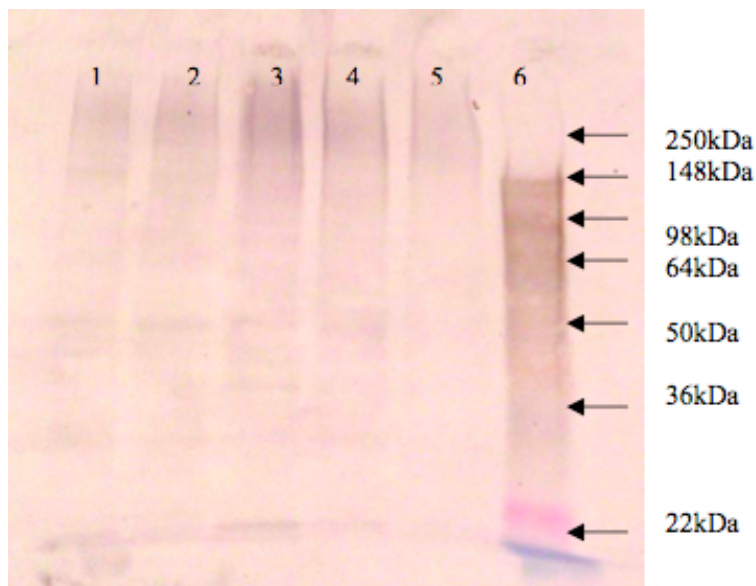
Figure 8. Mixed developmental stage N2 protein fractions probed with pre-immune serum. (From left) 02/11/04 cuticle protein; 02/11/04 total protein; 08/13/04 cuticle protein; 08/13/04 total protein; 08/13/04 cellular protein; Invitrogen SeeBlue® marker. From Mutetwa 2007.

Surface Mutants of *C. elegans*

Various mutations in *C. elegans* affect cuticle composition and expression. *Srf* mutants are characterized by their altered surface binding of lectin or antibody stains, with no change to cuticle morphology (Kramer, 1997). *Srf-6(yj13)* mutants are

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characterized by their expression of an L1 surface antigen at later developmental stages (Grenache *et al.*, 1996; Kramer, 1997; Olsen *et al.*, 2006). Other *srf* mutants, such as *srf-2* or *srf-3*, have cuticle features abrogated by the mutations (Politz *et al.*, 1990; Politz and Phillip 1992; Kramer, 1997).

Two other notable classes of surface mutants are *bah* and *bus* mutants. *Bah*, or *biofilm absent head*, mutants are resistant to formation of a biofilm on the head by the bacterium *Yersinia pestis* (Darby *et al.*, 2007). When exposed to a different bacterium, *Microbacterium nematophilum*, *bus*, or *bacterially unswollen*, mutants do not develop the tail swelling at the anal region that occurs in wild-type as a result of infection (Gravato-Nobre *et al.*, 2005). Studying these surface mutants will allow for comparison of nematomucin expression with wild-type expression.

PROJECT PURPOSE

Previous projects have examined the expression of proteins within the cuticle of *C. elegans* as a model for mucin expression in *T. canis*. This project examines the differences in glycoprotein expression of the *C. elegans* cuticle among surface mutant strains. Use of a single-peptide antibody directed against the F41G3.10-5 synthetic peptide was used to compare N2 wild-type protein expression with various surface antigens among surface mutant strains.

METHODS

Protein Samples

Protein samples were prepared by lysis of mixed-stage worms by sonication. This lysate became the total protein fraction. Centrifugation separated the cellular and cuticle protein fractions. Spinning down the total protein formed the cellular protein fraction, or the supernatant, and the cuticle, which was the pellet. Mutant strains used are shown in table 1. Two N2 (wild-type) preparations were used, N2_2007 prepared by Mutetwa (2007) and N2 (8.13.04), prepared by Chenying Guo (unpublished).

Table 1. Mutant strains used for comparison to wild-type.

Strain	Gene	Allele
AT18	*srf-6*	*(yj13)*
DC3	*srf-2*	*(br3)*
DC10	*srf-2*	*(br10)*
DC8	*bah-2*	*(br8)*
DC4	*bus-4*	*(br4)*
DC11	*bus-17*	*(br11)*
DC7	*bah-2*	*(br7)*
DC1	*bah-1*	*(br1)*

BCA Protein Concentration Assay

For each protein fraction, 10ul of the sample was combined with 90ul of acetone in an eppendorf tube. The tubes were capped and incubated at -20°C overnight to allow for protein precipitation. The following day, the tubes were centrifuged for 10 minutes at room temperature in a microcentrifuge at the maximum speed. The supernatant was carefully removed with a micropipette and the tubes air dried for one hour uncapped to evaporate any remaining acetone.

Using the *Pierce Micro BCA Protein Assay* kit, the working reagent was prepared by combining 25 parts Reagent MA, 24 parts Reagent MB, and 1 part Reagent MC. A

total volume of working reagent sufficient for all samples to be tested was prepared according to the following formula:

(# of samples + 9 BSA standards) x (# of duplicates) x (1mL/sample) = Total working reagent volume.

0.5mL of the working reagent was added to each unknown sample tube, mixed to dissolve the protein pellet, and then transferred to a labeled test tube. An additional 0.5mL of working reagent was then added in addition to 1.0mL of dH_2O for a final volume of 2.0ml. In another set of test tubes labeled A-I, BSA standard dilutions were made as in table 2.

Table 2. Dilution series of BSA protein standard for BCA protein concentration assay.

Tube	Diluent- dH_2O (mL)	BSA (mL) Initial conc. of 2.0 mg/mL	Final BSA Conc. (ug/mL)
A	1.80	0.20	200
B	3.60	0.80 of A	40
C	1.60	1.60 of B	20
D	1.60	1.60 of C	10
E	1.60	1.60 of D	5.0
F	1.60	1.60 of E	2.5
G	1.92	1.28 of F	1.0
H	1.60	1.60 of E	0.50
I	2.00	-----------	0

1.0mL of each standard was transferred to a new test tube and 1.0mL of working reagent added for a final volume of 2.0mL. Standard and sample tubes were incubated at 60°C for one hour. After incubation the tubes were cooled to room temperature. Absorbance values of each sample and standard were then measured at 562nm all within ten minutes to minimize change in the color of the reaction. For duplicates, values were

averaged, and a standard curve using these averages was generated in Microsoft Excel to interpolate sample concentrations.

SDS-PAGE

Protein samples were pipetted into respective eppendorf tubes so that tubes contained ~10ug of protein. However volumes did not exceed 10ul. If less than 10ul was pipetted, volumes were brought up to 10ul with dH_2O. In another eppendorf tube, 10ul of Invitrogen SeeBlue®Plus 2 marker were pipetted. 10ul of Laemmli sample buffer (see appendix) was added to each sample and marker tube.

Tubes were then capped and heated on a boiling water bath for five minutes. After boiling, tubes were momentarily centrifuged and loaded into separate lanes on a Bio-Rad Tris-HCl Ready Gel® (12% polyacrylamide). 20ul of each sample was loaded into the gel. The gel ran for one hour at 160V or 1hr. and 20 minutes at 120V. After the gel ran, migration distances of the standards in the marker lane were measured from the top of the resolving gel. Some gels were stained with Coomassie Blue to detect total protein. In this instance, the gel was placed in a container with staining solution (see appendix) and covered to incubate overnight at 4°C on a shaker.

The following day, the staining solution was poured off and the gel was soaked in destaining solution (see appendix) overnight at 4°C or for ~5 hours at room temperature. After destaining, the solution was poured off and the gel was stored in 7% Acetic acid at room temperature. An image of the gel on a white light box was taken using a digital camera. The camera lens was made level to the plane of the gel so as not to skew the image.

Western Blot

0.5mL eppendorf tubes were labeled (# of tubes = # of protein fractions + one marker). 10ul of each sample were pipetted into their respective tubes and 10ul of Laemmli buffer aliquotted into each tube for a total volume of 20ul. The tubes were boiled for 5 minutes and then spun momentarily in a microcentrifuge. Afterwards, 20ul of each sample and marker were then loaded into separate lanes on a Bio-Rad Tris-HCl Ready Gel® (12% polyacrylamide). The proteins were separated electrophoretically for 1 hour at 160V.

After electrophoresis, the transfer apparatus was set up, apparatus chamber filled with transfer buffer (see appendix), and a Millipore Immobilon P Polyvinylidene Fluoride (PVDF) transfer membrane (7.0cm x 8.4cm) prepared for transfer. To prepare the membrane, it was first soaked in methanol for 15 seconds, then dH$_2$O for 2 minutes, and lastly in transfer buffer for 5+ minutes. Fiber and sponge pads were also soaked in transfer buffer. A blotting sandwich was prepared using the cassette, a sponge pad, fiber pad, gel, prepared membrane, fiber pad, and sponge pad. The membrane was also cut to fit the size of the gel. This arrangement is shown in Figure 9.
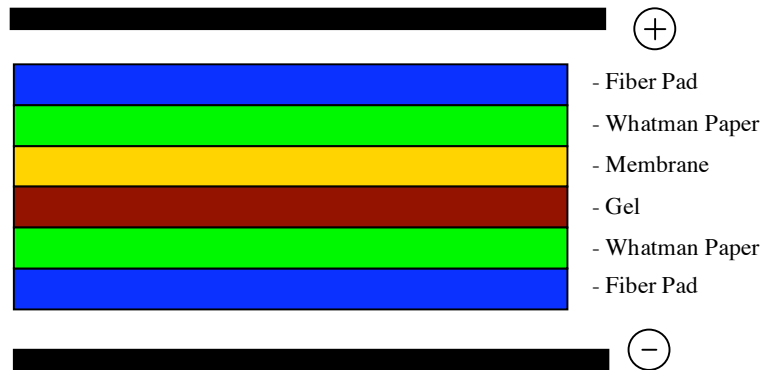
Figure 9. Cassette setup for transfer apparatus.

A stir bar was place in the chamber to equilibrate the temperature of the buffer throughout its volume. The transfer cassette was placed into the chamber and the transfer was performed for 1 hour at 120mA. The apparatus was placed in ice on a stir plate to maintain the temperature of the buffer.

After the transfer ran, the membrane was covered in 30mL blocking buffer (see appendix) overnight at 4°C on a shaker. The following day, the old blocking buffer was poured off and 30mL of fresh blocking buffer was poured onto the membrane along with 100ul primary antibody. This incubated overnight on a shaker at 4°C. The following day the primary antibody/blocking buffer solution was removed and stored in a 50mL conical tube at 4°C. The membrane was then washed with 50mL blocking buffer for 10 minutes on a shaker at room temperature (3x).

Blocking buffer was then poured off and 20mL fresh blocking buffer and 20ul secondary antibody (Sigma #F9259, goat anti-mouse IgM horseradish peroxidase) were poured onto the membrane and allowed to incubate on the shaker at room temperature. The buffer solution was poured off and the membrane was then washed again with 50mL blocking buffer for 10 minutes on a shaker at room temperature (3x). Color of the membrane was developed using the Bio-Rad Opti-4CN kit until color was satisfactory. One part Opti 4CN diluent and nine parts dH_2O for every $0.25cm^2$ of membrane were combined. 0.2mL of the substrate (per 10mL of diluent) were then added to the diluent mixture, mixed, and poured onto the membrane. When the color of development was satisfactory, development was stopped by placing the membrane in dH_2O for 2 minutes. Membranes were wrapped in a paper towel, stored out of light, and allowed to dry overnight. For long-term storage, membranes were placed in aluminum foil.

Spot Test

While a Millipore Immobilon P Polyvinylidene Fluoride (PVDF) transfer membrane was prepared, 10ul of each sample was pipetted into an eppendorf tube and boiled for five minutes. The membrane was then spotted with 10ul of each sample and sample spots allowed to dry. The membrane was then blocked with 30mL of blocking buffer for one hour at room temperature on a shaker. The membrane was cut into the appropriate number of divisions. For example, if two sets of tests were being run on the same membrane, the membrane was cut in half.

For a negative control and test sample, the membrane was cut in half, and one half incubated at room temperature for one hour in 20 ml blocking buffer, and the other half incubated at room temperature with 20mL blocking buffer and 66ul primary antibody, both on a shaker. The amount of primary antibody and blocking buffer was determined from the primary antibody/blocking buffer ratio in the western blot procedure (100ul primary antibody: 30mL blocking buffer), where 20mL blocking buffer was needed to cover the membrane sufficiently for incubation. The primary antibody solution was stored in a conical tube, and membranes were washed three times on a shaker for 10 minutes each with fresh 15mL blocking buffer each time. Both membranes were then incubated at room temperature for one hour with 30ul secondary antibody and 15mL blocking buffer on a shaker. Membranes were then washed again in the same manner three times.

Development of the membrane color occurred by combining one part Opti 4CN diluent and nine parts dH_2O for every $0.25cm^2$ of membrane. 0.2mL of the substrate (per 10mL of diluent) were then added to the diluent mixture, mixed, and poured onto the

membrane. When the color of development was satisfactory, development was stopped

by placing the membrane in dH$_2$O for 2 minutes. Membranes were wrapped in a paper

towel, stored out of light, and allowed to dry overnight. For long-term storage,

membranes were placed in aluminum foil.

RESULTS

Protein Concentrations

Concentrations of protein fractions prepared by Mutetwa (2007) are shown in

table 3. These were determined by Mutetwa by a BCA assay.

Table 3. Concentrations of mutant and wild-type protein fractions.
(Mutetwa, personal communication).

Genotype	Protein Sample	Concentration (ug/ul)
Wild type	Total	3.833
	Cellular	7.684
	Cuticle	7.061
srf-2(br3)	Total	2.714
	Cellular	4.805
	Cuticle	0.753
srf-2(br10)	Total	1.565
	Cellular	1.610
	Cuticle	0.402
	Total	5.887
	Cellular	1.105
	Cuticle	-----------------
srf-6(yj13)	Total	5.047
	Cellular	1.327
	Cuticle	1.258
bah-2(br8)	Total	6.055
	Cellular	5.347
	Cuticle	2.761
bus-4(br4)	Total	3.043
	Cellular	8.288
	Cuticle	1.049
bus-17 (br11)	Total	2.313
	Cellular	1.926
	Cuticle	0.076
bah-2(br7)	Total	2.131
	Cellular	5.480
	Cuticle	3.861
bah-1(br1)	Total	0.225
	Cellular	0.639
	Cuticle	0.367

Protein concentrations determined by a BCA assay for the N2 (8.13.04), N2_2007, *bah-1(br1)*, *srf-2(br3)*, and *srf-6(yj13)* protein fractions are shown in table 4.

Table 4. Concentrations of mutant and wild-type protein fractions.

Sample	N2 (8.13.04) (ug/ul)	N2_2007 (ug/ul)	*srf-6(yj13)* (ug/ul)	*bah-1(br1)* (ug/ul)	*srf-2(br3)* (ug/ul)
Total	7.140993789	0.21372549	12.19068323	0.238562092	N/A
Cellular	5.234161491	1.056862745	12.84161491	2.283660131	1.122875817
Cuticle	9.57826087	0.974509804	4.636645963	0.187581699	0.669934641

Viability of Protein Samples

Protein extracts from mixed stages of each strain were loaded in separate SDS-PAGE gel lanes. Sample viability and degradation were inferred from the intensity of the stainings. The intensities of the lanes in Figure 10 suggested the presence of similar sample concentrations among the *bah-1(br1)*, *srf-2(br3)*, and N2_2007 protein fractions, except for the cuticle fractions, which appeared to contain less protein than other fractions.
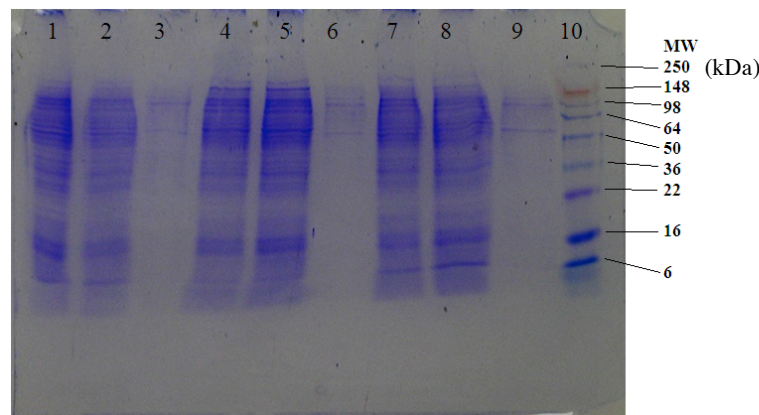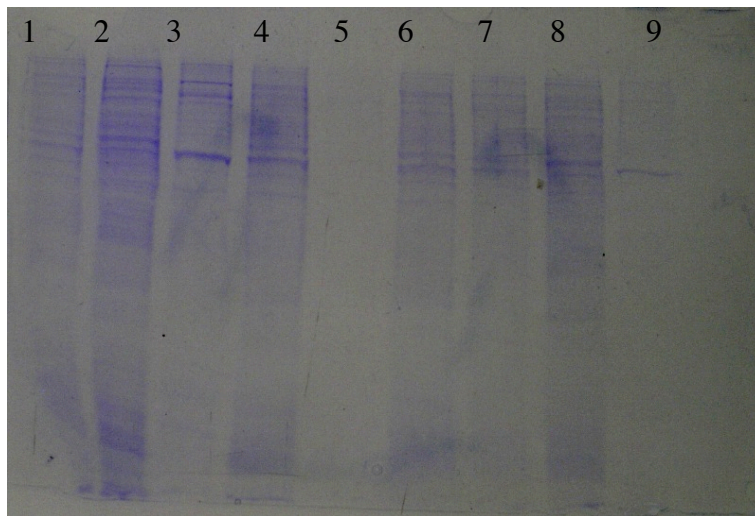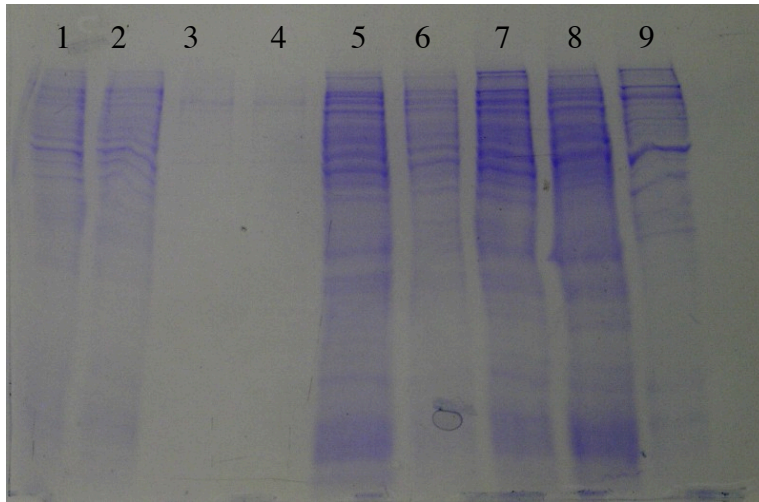

Figure 10. Comparison of protein concentrations by coomassie staining. Lane 1, *bah-1(br1)* Total, 2.4ug; Lane 2, *bah-1(br1)* Cellular, 10.0ug; Lane 3, *bah-1(br1)* Cuticle, 1.9ug; Lane 4, N2_2007 Total, 2.1ug; Lane 5, N2_2007 Cellular, 10.0ug; Lane 6, N2_2007 Cuticle, 9.7ug; Lane 7, *srf-2(br3)* Total, 27.1ug; Lane 8, *srf-2(br3)* Cellular, 10.0ug; Lane 9, *srf-2(br3)* Cuticle, 6.7ug; Lane 10, Invitrogen SeeBlue® marker.

Figures 11 and 12 examine the viability of *bus-4(br4)*, *bus-17(br11)*, *srf-6(yj13)*, *bah-2(br7)*, *srf-2(br10)*, and *bah-2(br8)* protein fractions. No marker is shown in Figures 11 and 12. The intensity of the stain for the *bus-17(br11)* cuticle fraction was weakest of all

fractions from *bus-4(br4)*, *bus-17(br11),* and *srf-6(yj13)*. The intensity of the stain of the

srf-6(yj13) cuticle fraction was more visible than the *bus-17(br11)* cuticle; however it

was weaker than the other fractions in the gel (Figure 11). The cuticle fractions of *srf-*

2(br10) and *bah-2(br7)* both stained the weakest compared to the total and cellular

fractions of the two strains. The staining patterns of the SDS-PAGE gels demonstrate that

the proteins are not grossly degraded, although this conclusion could not be reached

about the cuticle fractions, in which bands were poorly detected.

Figure 11. Comparison of protein concentrations by coomassie staining. Lane 1, *bus-4(br4)* Total, 30.4ug; Lane 2, *bus-4(br4)* Cellular, 82.9ug; Lane 3, *bus-4(br4)* Cuticle, 10.5ug; Lane 4, *bus-17(br11)* Total, 23.1ug; Lane 5, *bus-17(br11)* Cuticle, 0.76ug; Lane 6, *bus-17(br11)* Cellular, 19.3ug; Lane 7- *srf-6(yj13)* Total, 10.0ug; Lane 8, *srf-6(yj13)* Cellular, 10.0ug; Lane 9, *srf-6(yj13)* Cuticle, 4.6ug.

Figure 12. Comparison of protein concentrations by coomassie staining. Lane 1, *bah-2(br7)* Total, 21.3ug; Lane 2, *bah-2(br7)* Cellular, 54.8ug; Lane 3, *bah-2(br7)* Cuticle, 38.6ug; Lane 4, *srf-2(br10)* Cuticle, 4.02ug; Lane 5, *srf-2(br10)* Cellular, 16.1ug; Lane 6, *srf-2(br10)* Total, 15.7ug; Lane 7, *bah-2(br8)* Total, 60.6ug; Lane 8, *bah-2(br8)* Cellular, 53.5ug; Lane 9, *bah-2(br8)* Cuticle, 27.6ug.

Analysis of Protein Fractions

The single-peptide antibody directed against the unique sequence of the synthetic F41G3.10-5 peptide was used to probe the protein fractions to determine the presence of proteins containing this antigen. In the experiment shown in Figure 13, *srf-6(yj13)* and the two N2 preparations were compared. A band at ~52 kDa was present in most of the fractions; however, it was most prominent in the total and cuticle fractions of the N2 (8.13.04) preparation. The 52 kDa band was not detected in the N2_2007 total protein extract (Figure 13, lane 6). More notably, the 52 kDa band was present in the *srf-6(yj13)* total and cellular fractions, but was not detected in the *srf-6(yj13)* cuticle fraction.
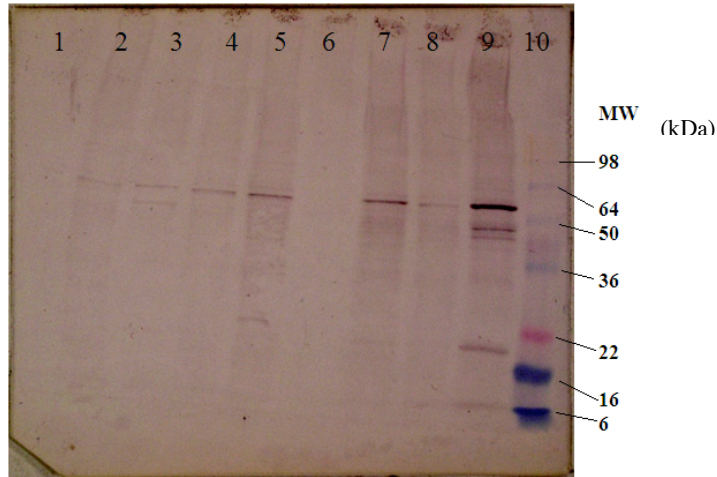
Figure 13. Western blot using the single-peptide antibody directed against the F41G3.10 protein. Lane 1, *Srf-6(yj13)* Cuticle, 10.0ug; Lane 2, *Srf-6(yj13)* Cellular, 10.0ug; Lane 3, *Srf-6(yj13)* Total, 10.0ug; Lane 4, N2_2007 Cuticle, 9.7ug; Lane 5, N2_2007 Cellular, 10.0ug; Lane 6, N2_2007 Total, 2.1ug; Lane 7, N2 (8.13.04) Total, 10.0ug; Lane 8, N2 (8.13.04) Cellular, 10.0ug; Lane 9, N2 (8.13.04) Cuticle, 10.0ug; Lane 10, Invitrogen SeeBlue® marker.

The experiment shown in Figure 14 compared the N2 (8.13.04) preparations with those of *srf-6(yj13)* and *srf-2(br10)*. Prominent presence of an ~52 kDa band was seen in the total and cuticle protein fractions of the N2 (8.13.04) sample. A less prominent band was also seen in the cellular N2 (8.13.04) fraction at ~52 kDa. In *srf-2(br10)* and *srf-6(yj13)* fractions, protein bands were not very visible.
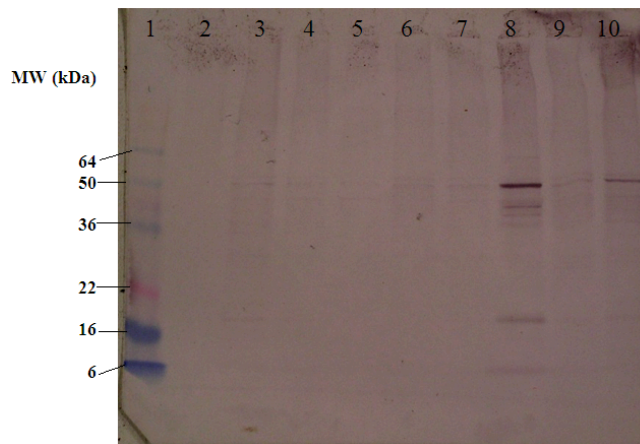
Figure 14. Western blot using the single-peptide antibody directed against the F41G3.10 protein. Lane 1, Invitrogen SeeBlue® marker; Lane 2, *srf-2(br10)* Cuticle, 4.02ug; Lane3, *srf-2(br10)* Cellular, 16.1ug; Lane 4, *srf-2(br10)* Total, 15.7ug; Lane 5, *Srf-6(yj13)* Cuticle, 10.0ug; Lane 6, *Srf-6(yj13)* Cellular, 10.0ug; Lane 7, *Srf-6(yj13)* Total, 10.0ug; Lane 8, N2 (8.13.04) Cuticle, 10.0ug; Lane 9, N2 (8.13.04) Cellular, 10.0ug; Lane 10, N2 (8.13.04) Total, 10.0ug.

N2_2007 fractions were then compared to *bah-1(br1)* and *srf-2(br3)* protein. (Fig. 15).

Here, prominent bands at ~52kDa appeared in the total and cellular fractions of *srf-2(br3)*, *bah-1(br1)*, and N2_2007. The N2_2007 total fraction, *bah-1(br1)* cuticle fraction, and *srf-2(br3)* cuticle fraction, however, did not reveal the 52kDa band. Additionally, a band at 36 kDa appeared in both the *bah-1(br1)* cellular and cuticle fractions as shown in Figure 15.
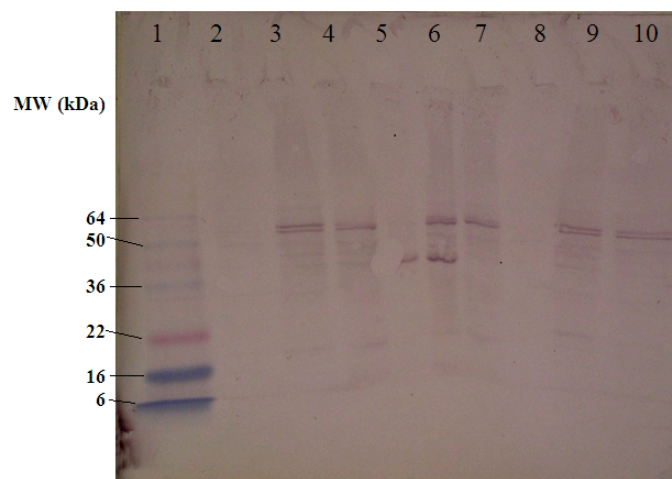

Figure 15. Western blot using the single-peptide antibody directed against the F41G3.10 protein. Lane 1, Invitrogen SeeBlue® marker; Lane 2, *srf-2(br3)* Cuticle, 6.7ug; Lane 3, *srf-2(br3)* Cellular, 10.0ug; Lane 4, *srf-2(br3)* Total, 27.1ug; Lane 5, *bah-1(br1)* Cuticle, 1.9ug; Lane 6, *bah-1(br1)* Cellular, 10.0ug; Lane 7, *bah-1(br1)* Total, 2.4ug; Lane 8, N2_2007 Total, 2.1ug; Lane 9, N2_2007 Cellular, 10.0ug; Lane 10, N2_2007 Cuticle, 9.7ug.

For *bah-2(br7)* and *bus-4(br4)*, the single-peptide antibody probe was used to compare expression of the antigenic protein in these two strains to N2_2007. Bands showed less prominently than in other blots (see Figure 16). Visible bands were observed in cellular fractions of each strain but not in other fractions.

Use of the N2 (8.13.04) preparation was resumed when compared to *bah-2(br8)* and *bus-17(br11)*. However, when these blots were developed, only weak bands in the total and cuticle fractions of the N2 (8.13.04) sample were present. No other fractions

displayed presence of any protein bands. This was performed twice to examine this

result. Figures 17a and 17b show the results of these two blots.
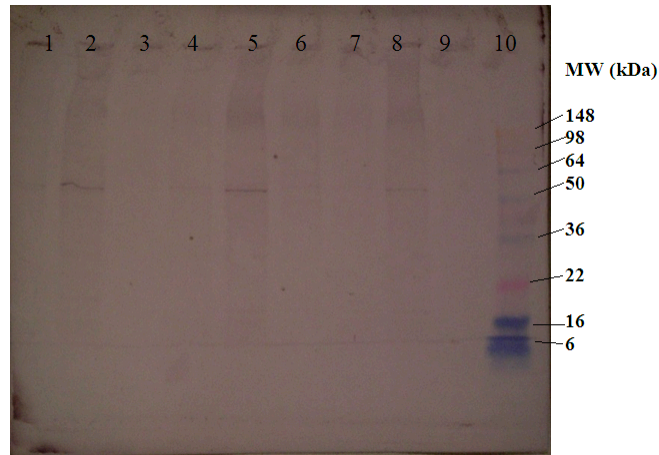

Figure 16. Western blot using the single-peptide antibody directed against the F41G3.10 protein. Lane 1, N2_2007 Total, 2.1ug; Lane 2, N2_2007 Cellular, 10.0ug; Lane 3, N2_2007 Cuticle, 9.7ug; Lane 4, *bus-4(br4)* Total, 30.4ug; Lane 5, *bus-4(br4)* Cellular, 82.9ug; Lane 6, *bus-4(br4)* Cuticle, 10.5ug; Lane 7, *bah-2(br7)* Total, 21.3ug; Lane 8, *bah-2(br7)* Cellular, 54.8ug; Lane 9, *bah-2(br7)* Cuticle, 38.6ug; Lane 10, Invitrogen SeeBlue® marker.
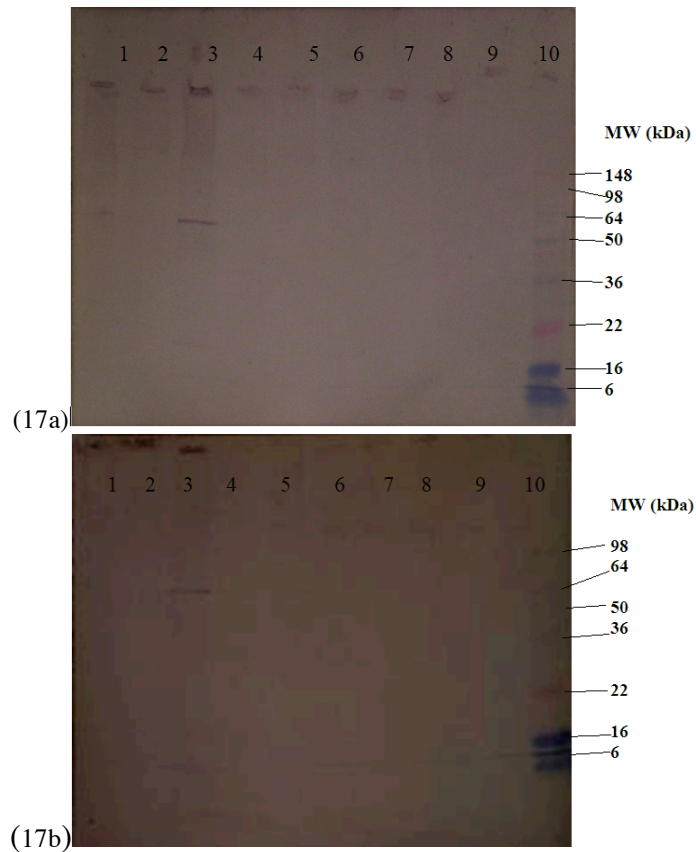
Figure 17. Western blot using the single-peptide antibody directed against the F41G3.10 protein. (Same for #a and #b): Lane 1, N2 (8.13.04) Total, 10.0ug; Lane 2, N2 (8.13.04) Cellular, 10.0ug; Lane 3, N2 (8.13.04) Cuticle, 10.0ug; Lane 4, *bah-2(br8)* Total, 60.6ug; Lane 5, *bah-2(br8)* Cellular, 53.5ug; Lane 6, *bah-2(br8)* Cuticle, 27.6ug; Lane 7, *bus-17(br11)* Total, 23.1ug; Lane 8, *bus-17(br11)* Cellular, 19.3ug; Lane 9, *bus-17(br11)* Cuticle, 0.76ug; Lane 10, Invitrogen SeeBlue® marker.

Integrity of Secondary Antibody

The activity of the secondary antibody used in the western blot procedure for the Figure 16 and Figure 17 blots was tested by a spot test. The concentration of the secondary antibody was tested in Figure 18, where the concentration was increased two-fold and three-fold. N2 (8.13.04) preparations were used, and after color development, no color appeared when the membrane dried.
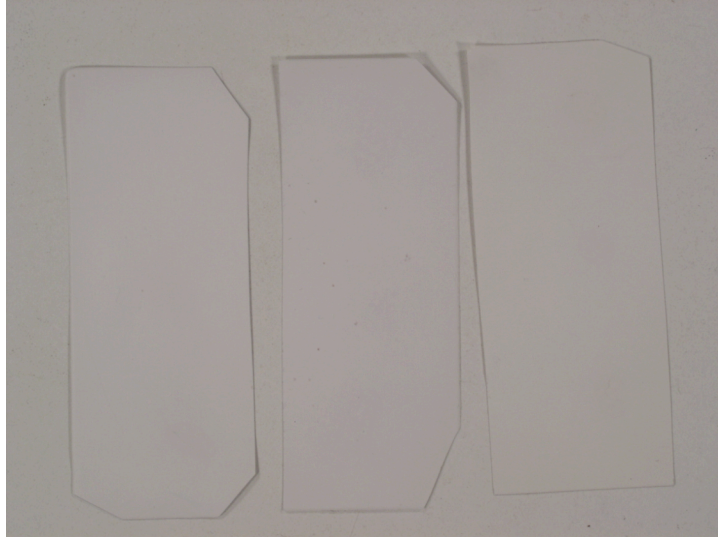
Figure 18. Spot test concentration assay. From left- three-fold, two-fold, and normal concentration of secondary antibody. For each membrane strip: Top- N2 (8.13.04) total, 10.0ug; Middle- N2 (8.13.04) Cellular, 10.0ug; Bottom- N2 (8.13.04) Cuticle, 10.0ug.

The test was repeated with a control in which primary antibody was not added. Once again after color development, while the membrane was still wet, both the negative control and membrane developed with primary and secondary antibodies showed similar color as shown in Figure 19.
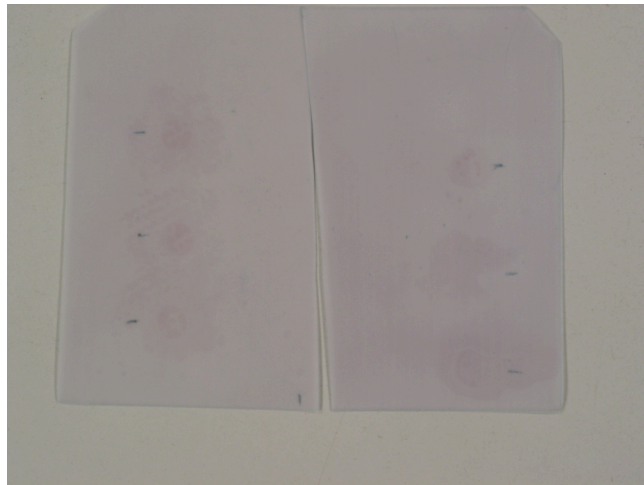

Figure 19. Spot test of secondary antibody. Left- negative control. Right- both primary and secondary antibody used. For each membrane strip: Top- N2 (8.13.04) total, 10.0ug; Middle- N2 (8.13.04) Cellular, 10.0ug; Bottom- N2 (8.13.04) Cuticle, 10.0ug.

DISCUSSION

Surface mutant protein samples were examined by SDS-PAGE and Coomassie staining to assess the viability of the samples. Due to an error in calculating protein concentrations, the protein amounts loaded for some mutant protein fractions were incorrect. This resulted in more than 10.0ug being used for some protein fractions in SDS-PAGE and western blot experiments. Conversely, less than 10ug of some mutant protein extracts was loaded because of low sample concentration. Observation of the protein concentrations in Tables 3 and 4 also indicates that some protein fractions contained very small protein amounts. Because of this, conclusions regarding protein viability and concentration of such samples are inconclusive. For the SDS-PAGE gels in Figures 11 and 12, the absence of the marker did not allow for any analysis of band sizes. Ultimately, the sample proteins were deemed undegraded since protein bands were visible in most samples, which made them suitable candidates for use in western blotting analysis. Weak band staining for cuticle fractions may be attributable to the collagen within these fractions not staining well with coomassie blue.

By using the single-peptide antibody, blots revealed antibodies bound to a specific 52 kDa antigen. The benefit of this method is that the antibody has specificity for the amino acid sequence specific to the protein as opposed to carbohydrates like lectin stains. For example, with a lectin stain, binding to multiple bands in cellular protein extracts is commonly observed. Western blotting procedures using this antibody were performed to compare the protein expression in surface mutants to protein expression in the wild-type N2 strain. The specificity of this antibody has revealed somewhat remarkable results. Firstly, some protein fractions in *bah-1(br1)*, *srf-2(br3)*, *bus-4(br4)*, *bah-2(br7)*, and *srf-*

6(yj13) displayed the presence of the F41G3.10 target peptide as demonstrated by the binding of the antibody to a protein at ~52 kDa similar to that of the wild-type N2 strain (Figures 13, 15 and 16). However, the ~52 kDa antigen was detected very weakly in *srf-6(yj13)* and *srf-2(br10)* extracts, in spite of the fact that protein amounts approximately equivalent to the positive controls were loaded (Figures 13 and 14). The other *srf-2* allele tested, *br3*, showed a more intense ~52 kDa band than did *srf-2(br10)*. These two alleles should be retested side-by-side and compared to other available *srf-2* alleles to determine if this difference is reproducible and extends to other mutant proteins. The *bah-1(br1)* strain also exhibited binding of the single peptide antibody to a protein band at 36 kDa in the cuticle and cellular fractions (Figure 15). This suggests that the expected 52 kDa protein may undergo post-translational processing such as proteolysis possibly due to defects in glycosylation, which normally helps to protect against this action. This would account for protein bands of two different sizes.

It was also noticed that in the wild-type N2 strain, a faint band appeared in the cellular fraction at 52 kDa much like the total and cuticle protein fractions (Figure 13). The predicted size of the F41G3.10 protein from its sequence is about 50 kDa (Fig. 4). Bands in the total and cuticle fractions are expected since the protein is secreted into the cuticle, and the total fraction also contains the cuticle fraction. An obvious explanation exists as a possibility for presence of the same protein in the cellular fraction. Protein processing occurs within the cell itself, not at the cuticle level of the worm. Therefore, it should be expected to see a protein band in the cellular fraction reflecting the presence of the protein en route to being secreted or having just been translated. Another reason why the 52 kDa band is observed in the cellular fraction could be contamination from the

cuticle fraction during preparation, or vice-versa. In addition, the protein being observed may be the underglycosylated form since glycosylation may prevent bind of the antibody to the target sequence.

There are various directions in which this project may proceed in the future. Firstly, the integrity of the later western blotting experiments was compromised, possibly a result of the secondary antibody. Blots comparing *bus-4(br4)*, *bah-2(br7)*, *bah-2(br8)*, *bus-17(br11)* to N2 did not develop well (Figure17), producing very faint bands for even the N2 control protein fractions when probed with the F41G3.10-5 single-peptide antibody. Comparison of these strains should be repeated for an accurate result. Mutetwa (2007) suggests examining the expression of protein products from all known surface mutants to compare glycosylation. In particular, *bus-4 and bus-17* encode a galactosyl transferase which would affect glycosylation (Hodgkin, personal communication). Moreover, since this project used mixed developmental stages when analyzing and comparing strains, differences in protein expression during development may have gone unnoticed. It is possible that the same protein is expressed throughout development yet is glycosylated in a different manner at each molt. Another possibility is an entirely different protein is expressed at each molt in the cuticle (Politz, personal communication). Various N2 stage worms may be compared for differences in development this way, or even use of mutant strains may be performed. Additionally, other mutants may be compared to N2 to determine similar protein expression.

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APPENDIX

Solutions

Blocking Buffer-

0.025M Tris-Base (pH adjusted to 7.5 with HCl), 0.15M NaCl, 1% BSA Fraction V, 0.1mM PMSF (5mM in EtOH from stock), 2mM EDTA (0.5M stock diNa·EDTA, pH 8.0). Sterile filter (0.45um pore) and store at 4°C.

Destaining Solution-

50% Methanol, 7% Acetic Acid

Laemmli Sample Buffer-

950ul Laemmli Sample Buffer, 50ul β-ME, store at 4°C.

Running Buffer-

Final concentrations: 25mM Tris-Base, 192mM Glycine (from solid salt), 0.1% SDS

Staining Solution-

0.1% Sigma-Aldrich EZBlue Coomassie Brilliant Blue G1041, 50% Methanol, 7% Acetic Acid

Transfer Buffer-

Final concentrations: 25mM Tris-Base, 192mM Glycine (from solid salt), 20% v/v Methanol, 0.025% SDS, resulting pH of 8.3. Store at 4°C.