Molecular & Biochemical Parasitology 165 (2009) 162–169

Contents lists available at ScienceDirect

Molecular & Biochemical Parasitology

Characterization of immunosuppressive surface coat proteins from *Steinernema glaseri* that selectively kill blood cells in susceptible hosts

Xinyi Li[a], E.A. Cowles[b], R.S. Cowles[c], R. Gaugler[d], D.L. Cox-Foster[a,*]

[a] Department of Entomology, The Pennsylvania State University, University Park, PA 16802, United States
[b] Department of Biology, Eastern Connecticut State University, Willimantic, CT 06226, United States
[c] Valley Laboratory, The Connecticut Agricultural Experiment Station, Windsor, CT 06095, United States
[d] Department of Entomology, The Rutgers University, New Brunswick, NJ 08901, United States

ARTICLE INFO

Article history:
Received 28 July 2008
Received in revised form 5 January 2009
Accepted 1 February 2009
Available online 10 February 2009

Keywords:
Entomopathogenic nematodes
Immune responses
Steinernema glaseri
Surface coat proteins
Hemocytes
Parasite

ABSTRACT

Surface coat proteins (SCPs) of entomopathogenic nematodes are implicated in the suppression/evasion of host immune responses, which is required for successful host colonization. *Steinernema glaseri* NC strain SCPs suppressed immune responses in oriental beetle larvae (*Exomala orientalis*), a susceptible host for *S. glaseri*, in a dosage-dependent manner, thus protecting *Heterorhabditis bacteriophora* from being killed in the same host. Melanization of *H. bacteriophora* decreased from 92 ± 5% in the untreated check to 1 ± 3% when protected by injection of 230 ng of *S. glaseri* SCPs. As the SCPs dosage increased, freely moving *H. bacteriophora* increased from 3 ± 4% in the untreated group to 57 ± 15% with an SCPs dose of 940 ng. At 2 h and in the absence of SCPs, 8% and 11% of hemocytes of *E. orientalis* were stained by propidium iodide and Hoechst, respectively. When exposed to 300 ng/μl SCPs, 70% and 96% were stained, respectively. At 6 h, propidium iodide stained 37% and 92% of the hemocytes without and with SCPs, respectively. In contrast, more than 90% of the cells were stained by Hoechst with or without SCPs. As native proteins, two isolated *S. glaseri* SCPs had an immunosuppressive effect; they were each composed of 38 kDa (PI = 4.6) and 56 kDa (PI = 3.6) subunits. SCP peptides were sequenced using LC–MS/MS and the mass fingerprints obtained with MALDI-TOF-MS; there were no significant matches found in peptide databases, which suggests that the SCPs studied are novel proteins. Twelve cDNA sequences were derived based on short peptides and 7 of them had no significant match against the *Caenorhabditis elegans* protein database. One of the cDNA matched an unknown *C. elegans* protein and the remaining 4 cDNAs matched proteins of *C. elegans* and *Brugia malayi*.

1. Introduction

Entomopathogenic nematodes in the genera *Steinernema* and *Heterorhabditis* are important biocontrol agents for soil dwelling insects [1,2]. These two groups of nematodes share a similar life history through convergent evolution and are closely related to vertebrate parasitic nematodes such as Strongylida and Rhadbditida (Strongyloididae) [3,4]. Entomopathogenic nematodes enter the hemocoel of the insect hosts via body openings and intersegmental membranes [4] and then release their symbiotic bacteria [5]. Symbiotic bacteria produce proteases and toxins that inhibit encapsulation, melanization and the production of antimicrobial peptides [1,6–8]. Since the sheath, a cuticle from previous molt that envelops infective juveniles (IJ), plugs the oral opening of the nematode and blocks the release of its symbiotic bacteria, the IJ has to shed it before, during or right after the penetration process of the infection [9,10]. The exsheathment has no effect on pathogenicity of some nematode species [9]. The exsheathment helps the IJs avoid immune recognition; however, the sheath is recognized by the host immune system [11].

Steinernema glaseri, originally isolated from Japanese beetle larvae (*Popillia japonica*) [12] releases its symbiotic bacteria, *Xenorhabdus poinarii*, 4–6 h after its entry of the host hemocoel [6,13]. *Heterorhabditis bacteriophora* is a commercialized biocontrol agent targeted for suppression of white grub populations [14]. Its symbiotic bacteria *Photorhabdus luminescens* can be detected as soon as 30 min after nematodes enter the hemocoel [15]. Bacteria are known to overwhelm host defenses 24–48 h after nematode infection, but within the first few hours of penetrating a host, the

Abbreviations: SCP, surface coat protein; PAGE, polyacrylamide gel electrophoresis; IEF, isoelectric *focusing*; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; LC–MS/MS, liquid chromatography/mass spectrometry/mass spectrometry.

* Corresponding author at: ASI building, Rm. 501, Department of Entomology, The Pennsylvania State University, State College, PA 16802, United States.
Tel.: +1 814 865 1895; fax: +1 814 865 3048.
E-mail address: dxc12@psu.edu (D.L. Cox-Foster).

0166-6851/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.molbiopara.2009.02.001

nematodes themselves are thought to manipulate the host immune response [16]. In mammals, parasitic nematodes have surface coat proteins (SCPs) that interfere with host immune responses; these proteins include antioxidant enzymes (glutathione peroxidase, superoxide dismutase, glutathione *S*-transferase and peroxiredoxin) and serine protease inhibitors (serpins) [17,18].

H. bacteriophora is encapsulated in *P. japonica* larvae; however, 10% of the nematodes escape the encapsulation after 24 h [6,7] by an unknown mechanism. In comparison, only 24% of *S. glaseri* are initially melanized in *P. japonica*; and after 24 h, all nematodes are free in the host hemocoel [6]. More than 75% of both *H. bacteriophora* and *S. glaseri* are recognized *in vitro* by hemocytes from susceptible wax worm (*Galleria mellonella*) hosts, semi-susceptible *P. japonica* hosts, and resistant house cricket (*Acheta domestica*) hosts [19]. In contrast, hemocytes of a host susceptible to *S. glaseri*, tobacco hornworm (*Manduca sexta*) initially only recognized 28% of these nematodes [19]. Therefore, nematodes may successfully infect hosts by either not being recognized by the host's immune system (e.g., *M. sexta*) or by evading the immune response following recognition (e.g., *Galleria* and *P. japonica*).

SCPs of *S. glaseri* are immunosuppressive and protect *H. bacteriophora* from immune responses in *P. japonica* and *Exomala orientalis* (the oriental beetle, another white grub species) but not in *M. sexta* [19,20]. The two SCPs lyse the hemocytes and suppress melanization in *P. japonica* [20]. Similar immune suppression of *G. mellonella* has been demonstrated from the cuticle of *Steinernema feltiae* [16]. Besides these initial studies, there is little information on the proteins involved in suppression or evasion of host immune response in insects.

In this research, the goal was to separate and characterize the *S. glaseri* NC strain SCP components that have immunosuppressive function. This may lead to a better understanding of the host immune response suppression mechanisms and the evolution of SCPs in parasitic nematodes.

2. Materials and methods

2.1. Nematodes and insects

S. glaseri (NC strain) and *H. bacteriophora* (HP88 strain) were cultured in last instar *G. mellonella* (Nature's Way Inc., Ross, OH) at room temperature. The IJ stage of nematodes were harvested, stored and washed before conducting experiments as described by Li et al. [19]. Last instar *P. japonica* and *E. orientalis* were field collected from Connecticut turf and maintained as described [19].

2.2. Injection

Ten *H. bacteriophora* IJs were delivered to the host hemocoel laterally to the base of a foreleg by a microinjector (Drummond Scientific Company, Broomall, PA) equipped with a glass needle [19]. Insect hosts were coinjected with either 4 μl Ringer's solution (0.8% sodium chloride, 0.02% calcium chloride, 0.02% potassium chloride, 0.02% sodium bicarbonate [pH 7.0]) or bovine serum albumin (BSA) solution (Sigma, St. Louis, MO).

2.3. SCP extraction

Several SCP extraction methods were compared. The SCPs were either extracted in cold 35% ethanol (−20 °C) or in 4 °C water, at 0 or 4 °C for 0.5 or 1 h, with IJ cuticle intact (with sheaths) or following desheathment (using 0.5% sodium hypochlorite at room temperature for 15 min then stored in aerated distilled water for 2 days) [19,20]. Proteins were also acetone-precipitated from water in which *S. glaseri* had been stored.

The following extraction procedure was most effective. Nematodes were washed thoroughly by filtration and added into 150 ml of cold 35% ethanol. The nematode/ethanol solution was stirred at low speed at 4 °C for 1 h. The filtrate from that suspension was frozen, lyophilized, and re-suspended in water, centrifuged at 14,000 × *g*, aliquotted and stored at −20 °C. The protein concentration was determined by modified Bradford assay [21,22].

2.4. SCPs dosage effect and exposure duration effect

To test the dosage effect of SCPs, 0, 50, 100, 230 and 940 ng of SCPs from *S. glaseri* were injected into *E. orientalis* with 10 *H. bacteriophora* IJs. IJs were recovered at 8 h post-injection.

To test the length of SCPs exposure on the immune response of the host, each larva was injected twice. For each group of 5 insects, at 1 h two insects were injected with Ringer's solution and the other three received injection of SCPs. After 8 h, one of the first two insects received another Ringer's injection, and two of the SCP-treated insects were injected with Ringer's and SCPs, respectively. IJs were recovered at 24 h after the first injection. The procedure of recovery of nematodes were as previously described [19]. The previous study has injected 5 ng of one component of SCP to study the immune responses [20], thus we used 100 ng of total SCPs so that results obtained will be comparable.

2.5. Effect of SCPs on E. orientalis hemocytes

The effect of SCPs on *E. orientalis* hemocytes was studied *in vitro* [23]. The larvae were surface sterilized before 2–4 drops of host hemolymph were collected [19] into 1.2 ml Grace's insect medium (Quality Biologicals, Gaithersburg, MD). Then the mixture was aliquotted into 4 wells of an agarose-coated coverglass bottomed chamber slides (Lab-Tek, Nunc, Naperville, IL) [19]. After adding 0.3 μg, 3 μg, 30 μg of SCPs or 30 μg of BSA into each well, the hemocytes were stained with Hoechst 33342 and propidium iodide (PI) following the manufacturer's protocol (Vybrant Apoptosis Assay Kit #5, Invitrogen, Carlsbad, CA). Six single-grub replicates were used in this experiment.

Observations with differential interference contrast (DIC) and fluorescence microscopy were carried out at 2 and 6 h after introducing the SCPs. Images were recorded using an inverted microscope with NIH Image [19]. The view was selected randomly; the number of total cells and cells stained by the Hoechst and PI in the same image were counted manually.

To test if the effects of SCPs on blood cells of insect hosts were related to the susceptibility of the hosts, similar procedures to those used for *E. orientalis* were used to stain the hemocytes from the northern masked chafer, *Cyclocephala borealis*. After 2 h incubation with or without 30 μg of SCPs, cells of *C. borealis* stained with SYBR14 dye and PI and cells of *E. orientalis* stained with Hoechst and PI were counted. The ratios of SYBR14 to PI and Hoechst to PI obtained for the respective species were compared using a *t*-test with 3–6 replicates.

2.6. SCPs isolation and characterization

S. glaseri SCPs in native buffer (50 mM Tris–Cl, 0.1% bromophenol blue, 10% glycerol, pH 6.8) and nondenatured protein molecular weight marker (Sigma, St. Louis, MO) were separated on 8% nondenaturing PAGE [19]. The outer lanes containing the molecular standards and total SCPs were excised and stained for 30 min at 37 °C (0.1% Coomassie Brilliant Blue R250, 50% methanol, 16% glacial acetic acid) [19]; while the inner portion of the gel was stored at 4 °C. Unstained gel strips corresponding to the major stained bands were excised and then electroeluted (Centrilutor, Millipore Inc.; Bedford, MA) according to the manufacturer's protocol. The eluants were

concentrated on 10 kDa-cutoff microconcentrators (Centricon, Millipore Inc.) at 4 °C, and washed with sterile ultrapure water to eliminate small molecules and salts. Aliquots of each fraction were stored at −20 °C. To test the effects of the eluted fractions, each was coinjected with *H. bacteriophora* into *E. orientalis*, allowed to incubate for 8 h, and the condition of the nematodes determined by dissection as previously described [19].

SCPa and SCPb were separated on 8% PAGE [19] with SDS-PAGE broad-range protein molecular weight markers (Bio-Rad, Hercules, CA) and native markers. For 2D PAGE, the first dimension slab IEF gel (without urea, pH 3.5–10.5) and 2D gels were run according to Cox and Willis [22]. Gels were stained with Coomassie blue [19] or silver stain [22]. For silver staining, the gel was fixed, washed 3 times in 50% ethanol for 20 min each, and submerged in 0.02% sodium thiosulfate solution for 1 min. After washing in water 3 times, the gel was incubated in silver nitrate solution (0.2% silver nitrate, 0.03% formaldehyde) for 20 min. The gel was washed and developed (3% sodium carbonate, 0.018% formaldehyde, 0.001% sodium thiosulfate) and development was stopped by 6% acetic acid for 6 min.

Glycosylation was analyzed by GelCode® Glycoprotein Staining Kit (Pierce, Rockville, IL) according to the manufacturer's directions.

2.7. MALDI-TOF-MS and LC–MS/MS sequencing of the proteins and cDNA sequencing

Protein spots were cut out from 2nd dimension PAGE after staining and gel pieces were destained in freshly-made destaining solution (15 mM potassium ferricyanide and 50 mM sodium thiosulfate for silver stain; 25 mM ammonium bicarbonate in 50% acetonitrile for Coomassie blue stain). Gel pieces were incubated in acetonitrile for 10 min, dried by SpeedVac and then incubated in 10 mM dithiothreitol in 100 mM ammonium bicarbonate for 1 h at 56 °C, 100 mM ammonium bicarbonate for 10 min and acetonitrile for 10 min sequentially. Gel pieces were next dried and rehydrated at 4 °C in digestion buffer (0.02 μg/μl trypsin (Promega Inc., Madison, WI) in 50 mM ammonium bicarbonate) for 45 min before overnight incubation at 37 °C. Last, the gel pieces were extracted (5% formic acid and 50% acetonitrile) three times and the eluant combined and dried to 10 μl for mass spectrometry (MS) analysis.

The mass fingerprints obtained by MALDI-TOF-MS (supplemental material II) were searched in Mascot using default parameters and significance set at $p < 0.05$ (http://www.matrixscience.com/cgi/search_form.plFORMVER=2&SEARCH=PMF).

Peptides longer than 10 amino acids derived from LC–MS/MS were blasted to wormbase (http://www.wormbase.org/db/searches/blast_blatusing.blastp) using blastp (protein query vs. protein database) and blastn (protein vs. nucleotide database) with default parameters. The database searched includes *Caenorhabditis elegans* (WS194) protein, *B. malayi* (assembly Bma1) proteins, *C. briggsae* (WS194) proteins, *C. elegans* (WS194) genome, *B. malayi* (assembly Bma1) genome, and *C. briggsae* (WS194) genome. Furthermore, a blastp (adjusted for short peptides automatically by the program) search was carried out in NCBI against non-redundant protein sequences from nematodes, using default parameters (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins&PROGRAM=blastp&BLAST_PROGRAMS=blastp&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on).

Degenerative primers were designed based upon the short peptide sequences obtained by liquid chromatography/mass spectrometry/mass spectrometry (LC–MS/MS) sequencing. Sequences included DDGTPMVE; DDVLAYT; DMDDDR; MDDDD; MSEMMAR and CNCWEL. Total mRNA was isolated from *S. glaseri* using TRIzol reagent (Invitrogen) and the first-strand cDNA synthesized by reverse-transcript polymerase chain reaction (RT-PCR) using M-MLV reverse transcriptase and random primers (Promega). The gradient PCR (annealing temperature from 50 to 65 °C) was used to generate potential cDNA sequences of interest. The PCR products were then cloned using a TOPO TA kit (Invitrogen) and sequenced. All procedures performed were as recommended by the manufacturers.

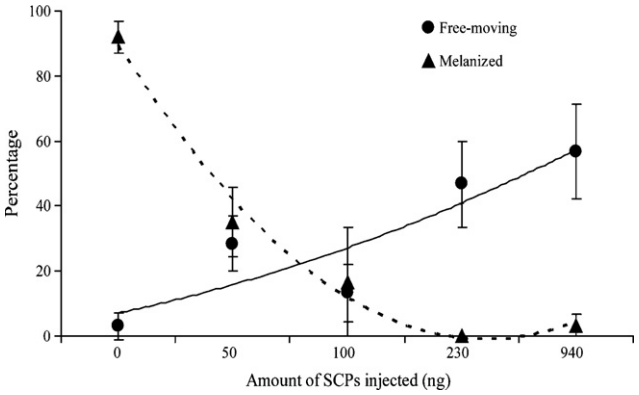

Fig. 1. Dosage effect of SCPs from *S. glaseri* on immune responses in *E. orientalis* larvae. *H. bacteriophora* ($N = 10$) were injected with 4 μl Ringer's solution or with various amounts of SCPs from *S glaseri*. Nematodes were recovered 8 h post-injection ($n = 9$–27).

2.8. Statistical analysis

The percentages of melanized and free-moving nematodes were calculated based upon the numbers of nematodes injected and means ± SE were graphed. Percentages were transformed to satisfy ANOVA assumptions where it was appropriate. ANOVAs were performed for each assay. If the results were significant, Tukey pairwise-comparisons or Dunnett comparisons were then performed. MINITAB 13.0 (Minitab Inc., State College, PA) was used for all analyses.

3. Results

3.1. SCP extraction comparison

Melanization of *H. bacteriophora* was greatly reduced by coinjection of *S. glaseri* SCPs, regardless of the extraction methods. The SCPs extracted at 4 °C without desheathment had the greatest specific activity and resulted in the lowest percent melanization (12 ± 7%). The proteins precipitated from storage water also suppressed melanization (data not shown).

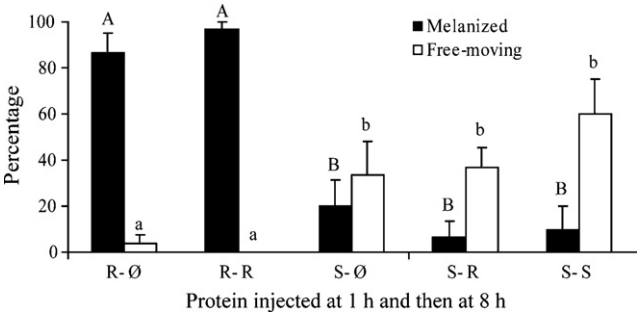

Fig. 2. Effect of exposure time of SCPs on immune responses in *E. orientalis* larvae. Two injections were made into each grub, one at 1 h and the second at 8 h. Code for injection treatments: R, Ringer's solution; Ø, no injection; S, injection of total SCPs. *H. bacteriophora* ($n = 10$) were injected with 4 μl Ringer's solution or with 230 ng of SCPs from *S glaseri* in Ringer's solution. Nematodes were recovered at 24 h after first injection. Means followed by the same letter are not significantly different (Tukey HSD test; $p = 0.05$; $n = 15$).

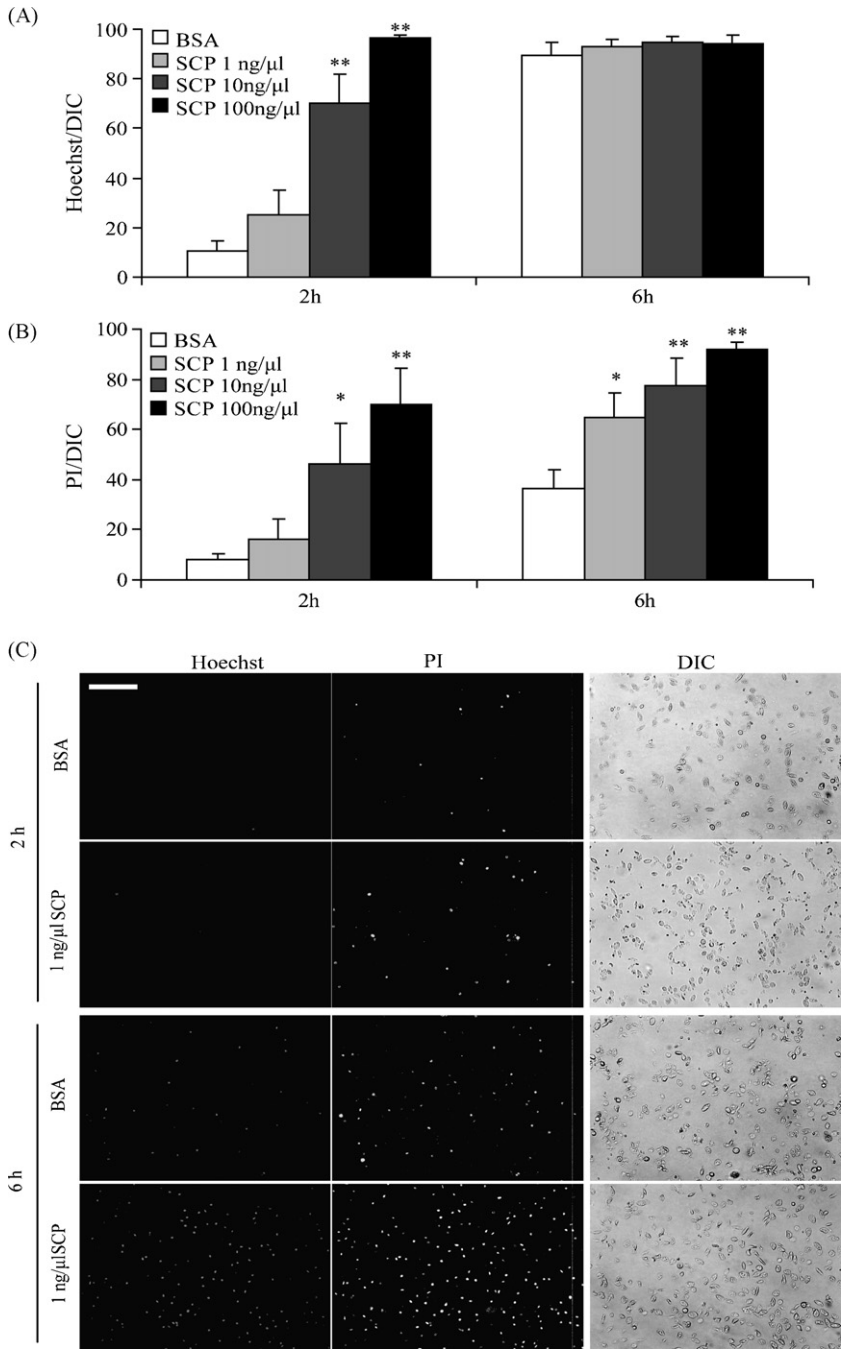

Fig. 3. Hoechst 33342 and propidium iodide (PI) *in vitro* staining of the *E. orientalis* larval hemocytes incubated with SCPs. Percentages of hemocytes stained by Hoechst (A) and PI (B) after incubation with BSA or SCPs (1, 10, or 100 ng/μl) for 2 and 6 h (ANOVA analysis shows significance for both PI ($F = 18.0$; df = 3, 35; $p < 0.001$) and Hoechst ($F = 21.6$; df = 3, 35; $p < 0.001$) stained cells at 2 hr. The symbols * ($p < 0.05$) and ** ($p < 0.01$) indicate statistical differences from the control (BSA group). (C) The images of hemocytes stained with Hoechst and PI at 2 and 6 h incubation with BSA and 1 ng/μl of SCPs. DIC, differential interference contrast image. The bar applies to all images and represents 100 μm.

3.2. SCPs dosage effect and exposure duration effect

More than 80% of *H. bacteriophora* were melanized when no SCPs were injected. In contrast, less than 40% of *H. bacteriophora* were melanized with coinjections of 50 and 100 ng of SCPs. When 230 and 940 ng SCPs were injected, very few *H. bacteriophora* were melanized in the insect host and about half of the nematodes were moving freely. ANOVA analysis of the data in Fig. 1 shows that increasing the amounts of SCPs injected into *H. bacteriophora* into *E. orientalis* resulted in decreasing melanization ($F = 29.48$; df = 4, 19; $p < 0.01$) and increasing percentage of freely moving nematodes ($F = 13.73$; df = 4, 19; $p < 0.01$) (Fig. 1).

The exposure to SCPs resulted in significant differences in melanized ($F = 13.73$; df = 4, 10; $p < 0.01$) and free-moving nematodes ($F = 9.63$; df = 4, 10; $p < 0.01$). Ringer's followed by no injection and the double Ringer's injection did not suppress melanization in *E. orientalis*. SCPs injection followed either by no injection or with Ringer's significantly inhibited melanization (Tukey; $t = 3.7$; $p < 0.03$) and about 30% of the nematodes were free-moving. When SCPs were injected twice, more than 50% of the nematodes were moving freely (Fig. 2). While the single and double SCP-injection groups do not significantly differ; these results suggest that the SCPs' immunosuppression effect can last more than 24 h inside *E. orientalis*.

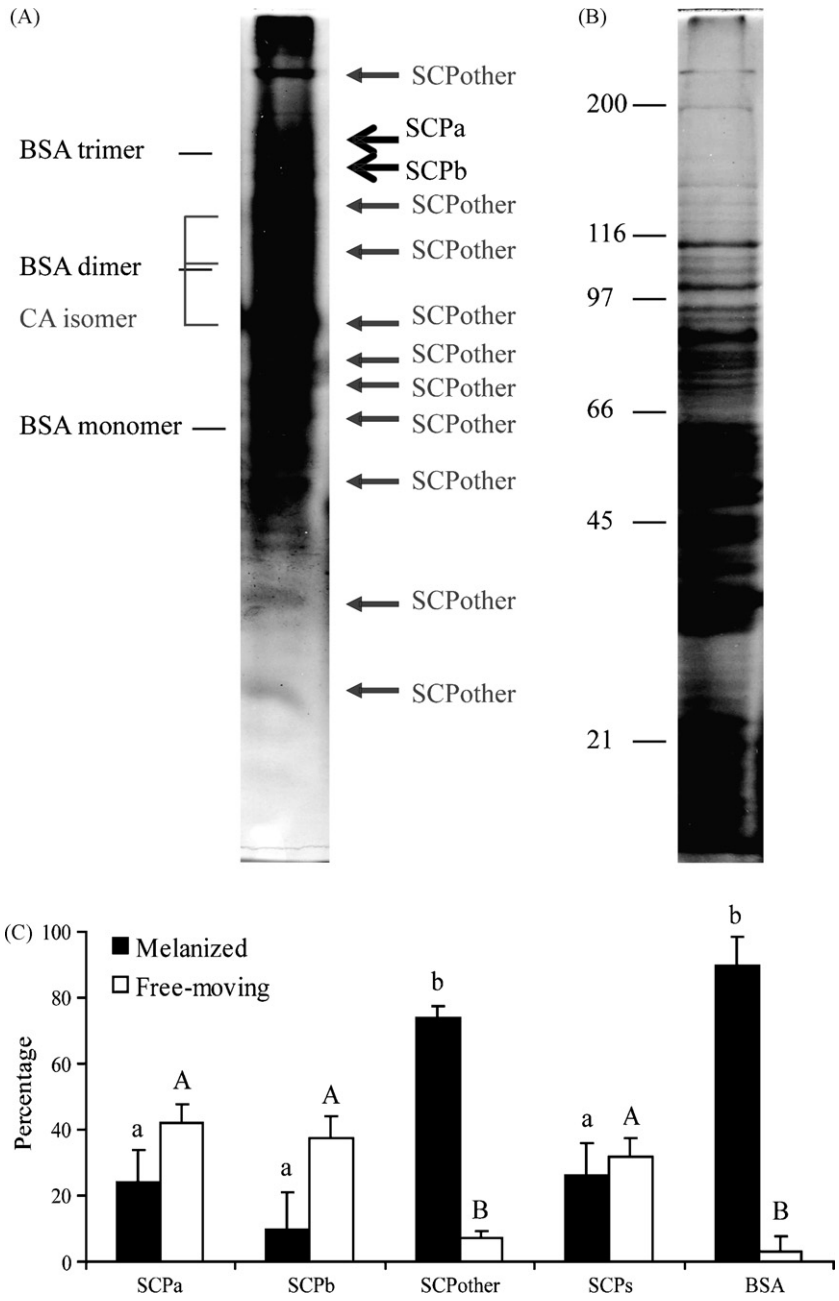

Fig. 4. *S. glaseri* SCPs (20 μg) on 8% nondenaturing PAGE (A) and 8% SDS-PAGE (B). Native protein standards were used for comparison among gels. Each marked band (arrow) on the native PAGE was electroeluted, concentrated and washed in microconcentrators. Electroeluted SCPa and SCPb (C) suppressed *E. orientalis* larval immune responses. *H. bacteriophora* ($n = 10$) were injected with 4 μl Ringer's solution, SCPa (120 ng), SCPb (300 ng), other isolated SCPs (SCP other) (80–1200 ng), total SCPs (230 ng) or BSA (600 ng). Proteins other than SCPa and SCPb were each electroeluted from fractions, the proteins were tested separately, and data were pooled. Nematodes were recovered 8 h after injection. Bars with the same letter do not significantly differ (Tukey HSD; $p = 0.05$; $n = 4$–36).

3.3. Effect of SCPs on E. orientalis larvae hemocytes

Blue fluorescent Hoechst dye can stain the chromatin of both living and dead cells, while PI permeates only dead cells (Fig. 3C). When *E. orientalis* hemocytes were exposed to a higher concentration of SCPs for 2 h, more hemocytes were stained by both PI and Hoechst than controls (Fig. 3A and B). For example, 8% of hemocytes were stained by PI with BSA incubation, while 16% and 70% of hemocytes were stained by PI with 1 and 100 ng/μl of SCPs treatment, respectively (Fig. 3B). After 6 h, more than 90% of the hemocytes were stained by Hoechst under each treatment (Fig. 3A). However, higher percentages of hemocytes were stained by PI when exposed to higher concentrations of SCPs ($F = 12.5$, df = 3, 23; $p < 0.001$). Only 37% of cells were stained by PI with BSA incubation, in comparison, 65% of the cells were stained by PI when incubated with 1 ng/μl of SCPs and 92% of the cells were stained by PI with 100 ng/μl SCPs (Fig. 3B). With increased incubation time, most of the hemocytes were stained by Hoechst, and the number of cells stained by PI was increasing. These results suggest that percentage of dying/dead cells is increasing over the time and in an SCP-dosage-dependent manner. The hemocytes of *C. borealis*, a resistant host of *S. glaseri*, were stained with SYBR14 dye, a similar dye to Hoechst, to compare the effect of SCPs on the blood cells from different species. With or without SCPs incubation, the ratio of cells stained with SYBR14 to the cells stained with PI *in C. borealis* were lower than the ratio of cells stained with Hoechst to PI in *E. orientalis* (ANOVA, $F = 9.56$,

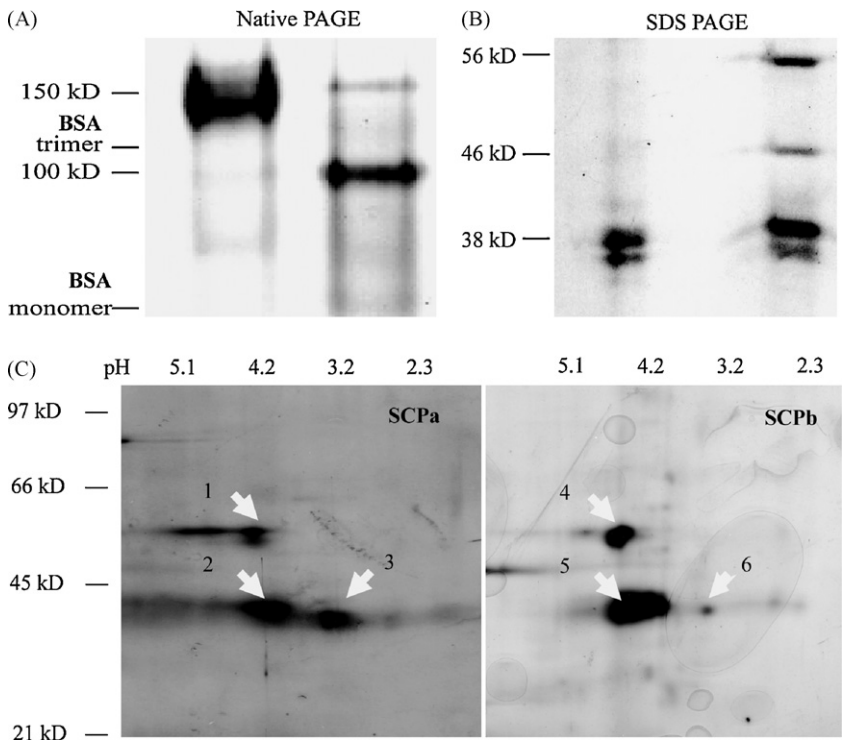

Fig. 5. Separation of SCPa and SCPb by nondenaturing PAGE (A), SDS PAGE (B) and 2D PAGE (C). SCPa and SCPb (6 μg each) were separated on 8% nondenaturing PAGE and SDS PAGE. Molecular weight on nondenaturing PAGE was estimated with nondenaturing standards. SDS PAGE indicated that both eluted bands were composed of smaller proteins. SCPa and SCPb (3 μg each) were separated in the first dimension by IEF PAGE (pH 3.5–9.5), then by 8% SDS-PAGE, followed by silver staining. SCPa and SCPb were each composed the three major proteins.

df = 1, 14, $p < 0.05$). Incubation of hemocytes with 100 ng/μl SCPs had similar effects in both species, with the ratios decreased in both *C. borealis* (0.24 ± 0.07 to 0.17 ± 0.03) and *E. orientalis* (0.95 ± 0.28 to 0.73 ± 0.15).

3.4. SCPs isolation and characterization

The *S. glaseri* SCPs were separated by both nondenaturing PAGE and SDS-PAGE (Fig. 3). The total SCPs were found to be quite complex in composition since there were many protein bands on native PAGE, and the same sample had even more protein bands on SDS-PAGE. This indicated that some larger molecular weight native proteins were multimeric and composed of smaller proteins (Fig. 4A and B). Extensive glycosylation was not detected for the majority of SCP bands; however, a few minor bands (not including SCPa and SCPb, described below) reacted with the periodic acid Schiff reagent (PAS) (data not shown).

S. glaseri SCPs were separated by nondenaturing PAGE and major bands were electroeluted and concentrated (Fig. 4A). Each fraction was tested against *E. orientalis*. Two fractions, SCPa and SCPb, protected *H. bacteriophora* from melanization and increased the percentages of free moving nematodes (Fig. 4C). Both SCPa and SCPb had the same immunosuppressive effect as the total extract. Other proteins electroeluted from the SCP extract did not suppress the immune responses in the host. Similarly, BSA delivered at the same concentration did not inhibit the host immune response; more than 80% of the nematodes were melanized and less than 10% of nematodes were free moving (Fig. 4C). The results indicated that the suppression of host immune responses was caused by injection of specific SCPs, SCPa and SCPb.

SCPa and SCPb were separated on nondenaturing PAGE (Fig. 5A) and SDS-PAGE (Fig. 5B). The estimated native molecular weight for SCPa was 150 kDa and for SCPb was 100 kDa (Fig. 5A). Given that a nondenaturing PAGE separates proteins based on molecular weight, charge and conformation, the native PAGE only approximates the size of the SCPs.

When SCPa and SCPb were denatured and separated by SDS-PAGE, both of the proteins were composed of smaller polypeptides (Fig. 5B). Each shared a major band of approximately 38 kDa. Native SCPa and SCPb were separated by IEF PAGE and followed by the 2nd dimension SDS-PAGE. The results indicated that both SCPa and SCPb were composed of three major proteins (Fig. 5C, spots 1–6). Approximately, spot 1 and spot 4 had a p*I* of 4.2 and were 54 kDa; spot 2 and spot 5 had a p*I* of 4.2 and were 40 kDa; and spot 3 and spot 6 had a p*I* of 3.6 and were 38 kDa (Fig. 5C), suggesting these proteins might be shared by SCPa and SCPb; spots 2, 3, 5 and 6 had the similar molecular weight and these spots probably were the two 38 kDa proteins on SDS-PAGE (Fig. 5C).

3.5. MALDI-TOF-MS and LC–MS/MS sequencing of the proteins and cDNA sequencing

The mass peak fingerprints (supplemental material II) obtained by MALDI-TOF-MS were searched in MASCOT peptide mass fingerprint (www.Matrixscience.com) per the updated database available; there were no significant matches (score of 78, $p < 0.05$) obtained. These results suggest that the isolated proteins are novel. Several short peptide sequences have been obtained using LC–MS/MS de novo sequencing (supplemental material I). The length of peptides obtained ranged from 7 to 19 amino acids. For example, PCWAQAVEVLESCGGHMDE and LSCPQAVEVCSMSEMMAR were obtained for spot 3. There is no significant homology found when we subjected the sequences (longer than 10 amino acids) for BLAST [24] against a nematode database. Although BLAST search in NCBI against nematodes indicated some positive hits with various *E*-values, there were no proteins sharing the same identity picked out by this approach.

We designed some degenerative primers based on the peptide sequences obtained, ran gradient PCR using cDNA derived from *S. glaseri* total RNA as template, and then cloned the products. After sequencing some of the positive clones (supplemental material III), cDNA sequences were subjected to BLAST against available nucleotide databases. Using wormbase blastx search (www.wormbase.org/db/searches/blast_blat), cDNA sequences obtained were searched against *C. elegans* and *Brugia malayi* protein database, and 7 of 12 sequences (supplemental material III, sequence Nos. 3, 5, 6, 7, 9, 10 and 12) have no significant matches with *E*-value set at the default threshold ($1E+0$), which indicates these sequences are species-specific cDNAs of *S. glaseri*. One cDNA sequence (No. 8) had a significant match to a putative protein in *C elegans* (Y41E3.1a CE32966 WBGene00012762, NCBI ID NP_001041024.1, score 236, $1E-60$) and in *B. malayi* (bma1.CDS.243745.0, NCBI ID EDP31608.1, score 236, *E*-value $1E-60$), which is a conserved hypothetical protein with unknown functions. cDNA sequence No. 1 matches a *C. elegans* gene encoding a protein containing a motif predicted to mediate protein–protein interactions. (EEED8.10a CE39344 WBGene00017138, score 57, $7E-09$ NCBI ID NP_001040757, in *B. malayi* bma1.CDS.252999.0, score 45, *E*-value $1E-05$). Sequence No. 2 matches phosducin-like protein required for G protein signaling in both *C. elegans* and in *B. malayi* (in *C. elegans*, Y62E10A.8 CE22697 WBGene00003142, score 50 *E*-value $1E-06$, in *B. malayi*, NCBI ID EDP34577.1, score 48.9, *E*-value $7E-09$,). cDNA sequence No. 4 matches The elo-3 gene encodes polyunsaturated fatty acid elongases in *C. elegans* (D2024.3 CE34783 WBGene00001241 score 28 *E*-value 0.75). cDNA sequence No. 11 is a homologue of a *C. elegans* gene encoding a neprilysin, thermolysin-like zinc metallopeptidase found on the outer surface of animal cells that negatively regulates small signaling peptides (e.g., enkephalin, tachykinin, insulin, and natriuretic peptides) by cleaving them (F54F11.2 CE11095 WBGene00010070 score 31 *E*-value 0.42).

4. Discussion

There are two main strategies to avoid recognition by the host immune system. One is immune evasion and the other is immune suppression. Both strategies always involve the parasite surface [25–29]. For example, nematodes that infect mammals secrete some glycoproteins that convey immuno-evasive effects [30,31].

The cuticle of *S. feltiae* is reported to play a role in inactivation of the pro-phenoloxidase pathway [16], an enzyme involved in the melanization process. The present research demonstrated that SCPs from *S. glaseri* suppressed the immune responses in *E. orientalis* larvae, protecting *H. bacteriophora* from being melanized. However, it failed to protect *H. bacteriophora* from *M. sexta* [19]. Different species of nematodes induce various immune responses in different insect hosts, which probably are correlated with the differences in surface coat proteins of the nematodes [19]. Even within *S. glaseri*, different strains vary greatly in immunosuppressive effect [19]. The *S. glaseri* immunosuppressive interaction appears to be quite host-specific. Here, we demonstrated a dosage effect of the SCPs on immunosuppression, and the effects were long lasting.

SCPa and SCPb each were immunosuppressive in *E. orientalis*. There was evidence that *E. orientalis* hemocytes were lysed 3 h after exposure to SCPa and SCPb (data not shown) [32]. Few hemocytes underwent apoptosis during our 6 h observation period. These results suggest that the SCPs kill the hemocytes; however, induction of apoptosis of the hemocytes may not be a major mechanism. We have also observed similar results when incubating hemocytes from *P. japonica* with SCPs in DAPI and PI dyes: increased percentage of cells stained by PI with increased incubation time and concentration of SCPs. In *C. borealis*, the staining by PI and Hoechst was not different between the hemocytes treated with and without SCPs indicating SCPs from *S. glaseri* NC effect susceptible hosts but not *C. borealis*, a resistant host [19]. These results suggest that SCPs have species-specific effects and that SCPs may suppress the immunity in susceptible hosts but not in resistant hosts.

Interestingly, both SCPa and SCPb bands of about 150 kDa separated into 3 smaller protein components on denaturing gels. SCPa and SCPb share one protein with identical molecular weight and p*I*, and other proteins of similar sizes with different p*I*'s. It is possible that the protein complex may be required for immunosuppressive function or the shared the protein is sufficient to carry out the function.

There is remarkable conservation in the biochemical and basic structure of cuticle among nematode groups, which includes free living *C. elegans*, parasitic nematodes infecting mammals and entomopathogenic nematodes [33]. All nematodes share a surface coat of glycocalyx composed of a few glycoproteins. The genome projects of *C. elegans* and a filarial parasite *B. malayi* have demonstrated extensive homology between these two species [33]. However, 35% of transcripts expressed in *B. malayi* have no homologues in *C. elegans* [33]. The greatest differences between the *C. elegans* and parasitic nematodes are species- and stage-specific surface proteins [33]. Using LC–MS/MS and MALDI-TOF-MS techniques, we have *de novo* sequenced the SCPs of interest from infective juveniles of *S. glaseri*; there were no homologies found from peptide sequences, protein mass finger prints and potential cDNA. These results suggest the SCPs of interest are novel and are not conserved among *C. elegans* and *B. malayi*. Given the entomopathogenic nature of our study nematodes, these SCPs may have evolved specific insect-immune system suppressing functions.

In the future, we expect that complete protein sequences and sequences of the encoding genes will help us to understand the evolution of SCPs among nematodes and shed light on the mechanisms nematodes use to counteract their hosts' immune systems.

Acknowledgements

Thanks go to Dr. Yi Wang who aided us in protocols previously used to analyze SCPs, to Dr. A. Daniel Jones who contributed valuable ideas and aided in protein analysis via mass spectrometry, and to Dr. Jianyong Li who facilitated protein sequencing and helped analyzed some samples. Thanks go to Dr. Stephen L. Rathbun for his statistical input. We also want to thank Dr. Chris Brey for his pilot work. Thanks go to Dr. Xiaolong Yang and Owen Thompson. Finally, we want to thank Dr. Mary Barbercheck for offering *S. glaseri* NC strain and Dr. Albrecht M. Koppenhöfer and Dr. Paul Heller for generously offering us beetle larvae.

This research was supported by USDA Grant (No. 02-35316-12255, "Genetic Engineering of Nematodes for Suppression of Insect Cellular Immune Response").

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molbiopara.2009.02.001.

References

[1] Dunphy GB, Thurston GS. Insect immunity. CRC Press; 1990.
[2] Kaya HK, Gaugler R. Entomopathogenic nematodes. Annu Rev Entomol 1993;38:181–206.
[3] Blaxter M, Dorris M, De Ley P. Patterns and processes in the evolution of animal parasitic nematodes. Nematology 2000;2:43–55.
[4] Burnell AM, Stock SP. Heterorhabditis, Steinernema and their bacterial symbionts—lethal pathogens of insects. Nematology 2000;2:31–42.
[5] Dunphy GB, Webster JM. Virulence mechanisms of *Heterorhabditis heliothidis* and its bacterial associate *Xenorhabdus luminescens*, in non-immune larvae of the greater wax moth *Galleria mellonella*. Int J Parasitol 1988;18:729–37.

[6] Wang Y, Campbell JF, Gaugler R. Infection of entomopathogenic nematodes *Steinernema glaseri* and *Heterorhabditis bacteriophora* against *Popillia japonica* (Coleoptera Scarabaeidae) larvae. J Invertebr Pathol 1995;66:178–84.
[7] Wang Y, Gaugler R, Cui LW. Variations in immune-response of *Popillia japonica* and *Acheta domesticus* to *Heterorhabditis bacteriophora* and Steinernema species. J Nematol 1994;26:11–8.
[8] Gaugler R, Lewis E, Stuart RJ. Ecology in the service of biological control: the case of entomopathogenic nematodes. Oecologia 1997;109(4):483–9.
[9] Campbell LR, Gaugler R. Effect of exsheathment on motility and pathogenicity of two entomopathogenic nematode species. J Nematol 1992;24:365–70.
[10] Campbell LR, Gaugler R. Mechanisms for exsheathment of entomopathogenic nematodes. Int J Parasitol 1991;21:219–24.
[11] Peters A, Gouge DH, Ehlers R, Hague NGM. Avoidance of encapsulation by *Heterorhabditis* spp. infecting larvae of *Tipula oleracea*. J Invertebr Pathol 1997;70:161–4.
[12] Steiner G. *Neoaplectana glaseri* n.g., n. sp. (Oxyuridae) a new nemic parasite of the Japanese beetle (*Popillia japonica* Newm.). J Wash Acad Sci 1929;19:436–40.
[13] Akhurst RJ, Dunphy GB. In: Beckage NE, Thompson SN, Federici BA, editors. Tripartite interactions between symbiotically associated entomopathogenic bacteria (*Xenorhabdus* spp.), nematodes (Steinernematidae and Heterorhabditidae), and their insect hosts. San Diego: Academic Press; 1993. p. 1–23.
[14] Gaugler R, Grewal P, Kaya HK, Smith-Fiola D. Quality assessment of commercially produced entomopathogenic nematodes. Biol Cont 2000;17:100–9.
[15] Bowen D, Rocheleau TA, Blackburn M, et al. Insecticidal toxins from the bacterium *Photorhabdus luminescens*. Science 1998;280:2129–32.
[16] Brivio MF, Pagani M, Restelli S. Immune suppression of *Galleria mellonella* (Insecta, Lepidoptera) humoral defenses induced by *Steinernema feltiae* (Nematoda Rhabditida): involvement of the parasite cuticle. Exp Parasitol 2002;101:149–56.
[17] Maizels RM, Balic A, Gomez-Escobar N, Nair M, Taylor MD, Allen JE. Helminth parasites—masters of regulation. Immunol Rev 2004;201:89–116.
[18] Maizels RM, Blaxter ML, Scott AL. Immunological genomics of *Brugia malayi*: filarial genes implicated in immune evasion and protective immunity. Parasite Immunol 2001;23:327–44.
[19] Li X, Cowles RS, Cowles EA, Gaugler R, Cox-Foster DL. Relationship between the successful infection by entomopathogenic nematodes and the host immune response. Int J Parasitol 2007;37(3–4):365–74.
[20] Wang Y, Gaugler R. *Steinernema glaseri* surface coat protein suppresses the immune response of *Popillia japonica* (Coleoptera: Scarabaeidae) larvae. Biol Cont 1999;14:45–50.
[21] Cox-Foster DL, Stehr JE. Induction and localization of FAD-glucose dehydrogenase (GLD) during encapsulation of abiotic implants in *Manduca sexta* larvae. J Insect Physiol 1994;40:235–49.
[22] Cox DL, Willis JH. The cuticular proteins of *Hyalophora cecropia* from different anatomical regions and metamorphic stages. Insect Biochem 1985;15(3):349–62.
[23] Lovallo N, Cox-Foster DL. Alteration in FAD-glucose dehydrogenase activity and hemocyte behavior contribute to initial disruption of *Manduca sexta* immune response to *Cotesia congregata* parasitoids. J Insect Physiol 1999;45:1037–48.
[24] Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol 1990;215:403–10.
[25] Dunphy GB, Webster JM. Interaction of *Xenorhabdus nematophilus* subsp *nematophilus* with the haemolymph of *Galleria mellonella*. J Insect Physiol 1984;30(11):883–9.
[26] Dunphy GB, Webster JM. Partially characterized components of the epicuticle of Dauer juvenile *Steinernema feltiae* and their influence on hemocyte activity in *Galleria mellonella*. J Parasitol 1987;73:584–8.
[27] Dunphy GB, Webster JM. Lipopolysaccharides of *Xenorhabdus nematophilus* (Enterobacteriaceae) and their haemocyte toxicity in non-immune *Galleria mellonella* (Insecta: Lepidoptera) larvae. J Gen Microbiol 1988;134:1017–28.
[28] Ratcliffe NA, Rowley AF, Fitzgerald SW, Rhodes CP. Invertebrate immunity—basic concepts and recent advances. Int Rev Cytol Surv Cell Biol 1985;97:183–350.
[29] Politz SM, Philipp M. *Caenorhabditis elegans* as a model for parasitic nematodes—a focus on the cuticle. Parasitol Today 1992;8:6–12.
[30] Maizels RM, Blaxter ML, Selkirk ME. Forms and functions of nematode surfaces. Exp Parasitol 1993;77:380–4.
[31] Haslam SM, Houston KM, Harnett W, Reason AJ, Morris HR, Dell A. Structural studies of N-glycans of filarial parasites conservation of phosphorylcholine-substituted glycans among species and discovery of novel chito-oligomers. J Biol Chem 1999;274:20953–60.
[32] Wang Y, Gaugler R. Host and penetration site location by entomopathogenic nematodes against Japanese beetle larvae. J Invertebr Pathol 1998;72:313–8.
[33] Burglin TR, Lobos E, Blaxter ML. *Caenorhabditis elegans* as a model for parasitic nematodes. Int J Parasitol 1998;28:395–411.