

Report on Short Term Scientific Mission

COST Action : COST 850 Biocontrol Symbioses
Working Group: WG2 Bioactive Molecules
Recipient: Damien O'Halloran
Organisation: National University Ireland Maynooth
Host Institute: Scottish Crops Research Institute
Period: 14th JAN 02 to 4th FEB 02

Introduction

The infective juveniles (IJs) of *H.bacteriophora* occur in soil where they actively seek out and infect insect larvae. In the insect haemocoel the nematodes release cells of a symbiotic bacterium *Photorhabdus* sp. (which the nematodes carry in their intestine). These bacteria multiply rapidly in the haemolymph of the insect and kill the insect within 48 hours.

The symbiont bacteria produce and secrete variety of insecticidal toxins, proteases and lipases. Some of these toxins have been cloned, patented and transferred into transgenic plants (French-Constant, Bowen, 1999). It is known that the nematode also contributes to the pathogenic process, but the genes that are involved have not been investigated.

Katherine Dolan (PhD thesis, NUI Maynooth 2001) has studied the timing and location of RNA synthesis in *H.bacteriophora* during the early stages of insect infection. She found that the most intense staining occurs in the pharyngeal glands. Pharyngeal gland secretions are known to be important in the pathogenesis of other animal and plant parasitic nematodes (Maizels *et al.*, 2000). We would like to identify and catalogue the genes that are secreted from the pharyngeal glands early in the infections process in *Heterorhabditis* using an EST approach. ESTs are single pass sequences of cDNA clones selected at random from libraries made from the life-cycle stage of interest. Since EST sequences correspond to expressed genes and do not include any non-coding sequences they offer a rapid route to the discovery of novel genes.

A small-scale EST programme, which was carried by Katherine Dolan as part of her PhD thesis, resulted in the discovery of a series of ESTs obtained from chitinases. The number and abundance of these sequences in the dataset is greater than that found in similar stages of other nematode species. Chitin is a structural polysaccharide that occurs in the exoskeleton and the gut lining of insects. Chitinases have been detected in several insect parasitic organisms including malaria parasites, trypanosomes, *Bacillus thuringiensis*, the fungi *Metarhizium* and *Beauveria* and filarial nematodes whose intermediate stages occur in insects. The prevalence of chitinases in our EST data suggests that chitinases may also play a role in the *H.bacteriophora* infection process. Several areas of work now need to be undertaken in order to confirm this function for these chitinase genes, in particular an analysis of their spatial expression patterns using *in situ* hybridisation and the isolation of full length transcript sequences for these genes.

The technique of *in situ* hybridisation is a very important tool in functional genomics and has been adapted for use with nematodes (De Boer *et al.*, 1998). This technique is in routine use in the host laboratory where it has been used with a variety of nematodes to examine spatial expression patterns of other genes involved in pathogenesis in plant parasitic nematodes (e.g. Veronica *et al.*, 2001).

Experience with this technique will be of benefit to the STSM applicant, not only for his work on chitinase genes but he will also be able to transmit his knowledge to this technique to other members of his home laboratory for use in the analysis of other genes in the *H.bacteriophora* EST data set and other projects on *H.bacteriophora* molecular genetics.

Procedure and Results

Preparation of Dig labelled DNA probes

Three chitinase clones (144, 202 & Evo) have been isolated from a cDNA library of *Heterorhabditis bacteriophora*. Firstly, primers were designed to amplify a region of ~200-250bp for each clone. These PCR products were then digoxigenin labelled in a linear PCR:

DEPC-H ₂ O	10.4µl
10x Supertaq buffer	2.0µl
10x DIG-dUTP/dNTP mix	1.5µl
10M Primer: Forward or Reverse	4.0µl
Supertaq polymerase	0.1µl
Template	2.0µl (ca. 17ng)
Total	20.0µl

Both sense and antisense probe were prepared with the following PCR conditions:

Step1	94°C	2min
Step2	94°C	15sec
Step3	55°C	30sec
Step4	72°C	4min
Step5	goto step 2, 34x	
Step6	72°C	4min
Step7	4°C	hold

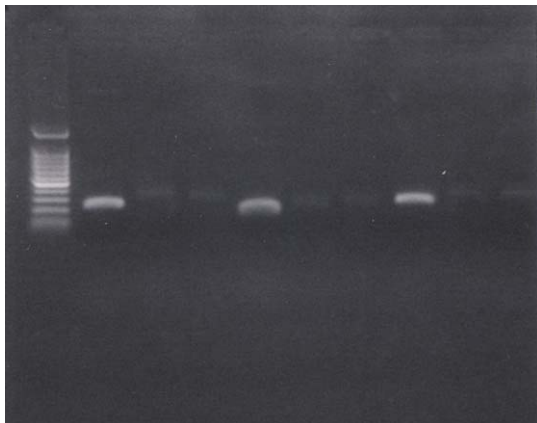


Fig. 1 Lane 1: 100bp marker. Lane 2: 144 template. Lane 3&4: 144 reverse and forward Dig labelled probe. Lane 5: 202 template. Lane 6&7: 202 reverse and forward Dig labelled probe. Lane 8: Evo template. Lane 9&10: Evo reverse and forward probe.

Recovery of Dauer Juveniles (DJs)

Newly emerged infective juveniles (IJs) were washed in 0.4% Hyamine and squashed between two sterile glass microscope slides, the exudate streaked out on NBTA plates. After 48 hours at 25°C dark green colonies were transferred to lipid agar plates and grown at 25°C for 48hrs. ~500 DJs were allowed to feed on lipid agar plates containing primary phase, *Photorhabdus luminescens* bacteria, for six hours and then washed with M9.

'In situ' hybridisation protocol

Recovered IJs were fixed in 2% paraformaldehyde for 24 hours at 4°C. The fixed IJs were washed in diluted fixative (1/10) and cut using a blade taped to a vibrating aquarium pump until most nematodes were severed in two or three pieces. Nematodes were then collected in a 1.5ml microfuge tube.

[The following incubations are with the nematode sections rotating in a 1.5ml RNase-free microfuge tube. Centrifugations are at room temperature at 8000rpm for 2min. All glassware baked and reagents made up with DEPC-treated dH₂O]

- Nematode sections were washed twice with 1ml of M9 buffer
- followed by incubation in 0.5ml Proteinase-K solution (0.5mg/ml) for 5 minutes rotating at 22°C.
- Nematode sections were then washed with 1ml M9 buffer and frozen on deep frozen ice for 15min.
- Suspend nematodes in 1ml of cold (-20°C) methanol and incubate for 30sec on deep frozen ice.
- Pellet nematode sections at 13000rpm for 30sec
- Suspend nematodes in 1ml of cold (-20°C) acetone and incubate 1min in deep frozen ice.
- Pellet nematodes at 13000rpm for 1min
- Remove acetone until 100µl is left in the tube
- Rehydrate nematodes by adding drops of DEPC-treated ddH₂O to the acetone until the volume is 200µl
- Spin the nematodes and remove the supernatant
- Wash the nematode sections with 500µl hybridisation buffer to remove the acetone
- Suspend nematodes in 1.2ml of fresh hybridisation buffer and aliquot 200µl into 0.5ml eppendorff tubes
- Pre-hybridise the nematodes for 15 min at 50°C
- Heat denature the DNA-probes for 10 min in a becker glass with dH₂O, cool them directly on ice
- Add 10µl of probe into each of the six 0.5ml eppendorffs
- Hybridise o/n at 50°C
- Wash three times for 15 min with 4xSSC at 50°C
- Wash three times 20 min with 0.1x SSC/0.1%SDS at 50°C
- Wash the nematodes once with maleic acid buffer (1min)
- Incubate the nematodes for 30min in 1% Boehringer blocking reagent in maleic acid buffer
- Incubate the nematodes for 2hours in alkaline-phosphate conjugated anti-digoxigenin antibody (Fab fragment) diluted 1:1000 in 1% Tween-20
- Wash briefly in alkaline detection buffer
- Stain nematode sections in Nitro Blue tetrazolium/X-phosphate staining solution o/n at 4°C.
- Stop the reaction by washing the nematodes twice in 0.01% Tween-20 in sterile H₂O
- Spin down nematodes and remove supernatant until ~50µl is left in the microfuge tube. Resuspend nematodes with a pipette and apply ~16µl to a microscope slide. Cover with a 40x22mm cover slip. Seal edges with nail varnish. Slides were examined under a light microscope.

This system had been optimised in the host laboratory for the potato cyst nematode, *Globodera rostochiensis*. During the short-term scientific mission I found that much optimisation was required to adapt the system for *Heterorhabditis bacteriophora*. The principal concern was regards the Proteinase-K treatment. The original protocol required 30 minutes incubation, however this proved too long and over-digested the nematodes. Finally a five-minute incubation was found to be sufficient to allow probe penetration and leave the internal structures visible. From this, faint staining was observed in the pharyngeal region. Further optimisation is currently being carried out in the home laboratory to enhance this staining. More probe will be used and longer fixation times allowed to permit more permeabilisation by the Proteinase-K. The temperature of the hybridisation and/or stringency washes may also be too high at 55°C and may require reduction. Centrifugation speeds were also increased to better final yields and sensitive cutting was required to keep intact the nematode structure. Chitinase genes may also be lowly expressed and may therefore require a more sensitive system of detection such as the signal amplification kit (TSA Direct) from NEN-Dupont. This kit also utilises an anti-DIG alkaline-phosphatase conjugated treatment of detection, which is the same as that described in the current protocol described above.

cDNA analysis

The three candidate chitinase ESTs obtained to date are derived from the 3' end of each gene. mRNA was isolated from recovered IJs and reverse transcriptase used to generate first-strand cDNA. Approximately 80% of nematode genes are linked to a 3' trans-spliced SL1 sequence. Using primers designed from the SL1 sequence second strand cDNA containing the 5' upstream coding sequence from the clones of interest was successfully amplified during the visit. This product is now undergoing sequencing and analysis.