

## Isolation of antibacterial response genes from the dung beetle *Copris tripartitus* (Coleoptera: Scarabaeidae) immunized with *Escherichia coli*

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**Abstract.** Dot blot hybridization of total RNA from normal and bacteria-injected larvae of the dung beetle *Copris tripartitus* identified 13 cDNA clones that seem to be associated with the immune response. A cDNA encoding a dung beetle homolog of the heat shock protein HSP70 (CTHSP70) was characterized by full-length sequencing and expression pattern analysis. CTHSP70 was upregulated 4 h after bacterial injection, reached maximum level after 8 h, and declined gradually after 16 h. Our data suggest that CTHSP70 and another 12 genes are involved in the anti-microbial defense that is particularly important for dung beetle larvae living in pathogen-rich conditions.

### INTRODUCTION

To defend themselves against infection by a variety of bacteria, fungi and parasites, insects have evolved several efficient defense mechanisms, including the production of antimicrobial compounds. So far, more than 200 antibacterial peptides have been identified in insects. These peptides are known to play important roles in the humoral defense reaction (Hultmark, 2003; Lemaître, 2004; Hoffmann, 2003; Hoffman et al., 1999; Engstrom, 1999; Garcia-Orozco, 2005). Humoral reactions include synthesis of antimicrobial proteins, which are secreted into the haemolymph, and proteolytic cascades leading to localized coagulation (Hoffman, 1995). The insect antibacterial peptides are classified into three groups, which are linear alpha-helical peptides devoid of cysteine residues, peptides in which proline and/or glycine residues are over-represented, and cysteine-rich peptides with cysteine established  $\alpha$ - $\beta$  motif (Bulet et al., 1999). *Copris tripartitus* spends a lot of time in dung, which contains an abundance of pathogens. The larvae feed on the fungi, decaying organic matter, dung and other organic materials in dung balls. Thus, it is likely that *C. tripartitus* defends itself against invading pathogens by means of antimicrobial compounds. Therefore, the purpose of the present study was to isolate and identify immune related genes in the dung beetle, *C. tripartitus*. In this study, we cloned immune-related genes differentially expressed in control and immunized larvae, and detected the time course of expression using Northern blot.

### MATERIAL AND METHODS

#### Immunization

To induce antibacterial peptides, the *Copris tripartitus* larvae were cooled on ice and individually injected with 50  $\mu$ l of *E. coli* JM109 ( $5 \times 10^5$  cells) suspended in physiological saline (150 mM NaCl/5 mM KCL). Larvae were analyzed after 0–24 h.

#### Construction of the full-length enriched cDNA library

cDNA library from larvae of *C. tripartitus* immunized with *E. coli* was constructed using a modification of Maruyama and Sugano's method (Maruyama & Sugano, 1994). Briefly, 100  $\mu$ g of total RNA was treated with 3 units of bacterial alkaline phosphatase (TaKaRa, Shiga, Japan) in 100  $\mu$ l of 100 mM Tris-HCl (pH 7.5), 2 mM DTT and 80 units of RNasin (Promega, Madison, WI, USA) at 37°C for 60 min. After phenol extraction and ethanol precipitation, the total RNA was treated with 100 units of tobacco acid pyrophosphatase (Waco, Tokyo, Japan) in 100  $\mu$ l 50 mM sodium acetate (pH 5.5), 5 mM EDTA, 10 mM 2-mercaptoethanol and 80 units RNasin at 37°C for 60 min. The pretreated total RNA was then ligated with 0.4  $\mu$ g of 5-oligoribonucleotide (5-oligo: 5-AGC AUC GAG UCG GCCC UUG UUG GCC UAC UGG-3) using 250 units of RNA ligase (TaKaRa) in 100  $\mu$ l of 50 mM Tris-HCl (pH 7.5), 5 mM MgCl<sub>2</sub>, 5 mM 2-mercaptoethanol, 0.5 mM ATP, 25% PEG 8000 and 100 units of RNasin at 20°C for 3 h. After completing these oligo-capping reactions, mRNA was isolated using a commercial kit, QIAGEN Oligotex<sup>TM</sup> (Qiagen, Hilden, Germany). The synthesis of first-strand cDNA from the purified mRNA, and the cDNA amplification were performed as described by Maruyama & Sugano (1994). The amplified PCR products were digested with *Sfi*I, and cDNAs longer than 1.3 kb were ligated into *Dra*III-digested pCNS-D2 in an orientation-defined manner. The pCNS-D2 vector contains 5 *Eco*RI-*Dra*III-*Eco*RV-*Dra*III sites at multi cloning sites, which was achieved by modifying the pCNS vector (GenBank Accession no. AF416744). The ligated cDNA was transferred into *E. coli* Top10F' (Invitrogen, Carlsbad, CA, USA) by electroporation (Gene Pulser II, Bio-Rad, Hercules, CA, USA).

#### Dot blot analysis and DNA sequencing

A total of 1862 random cDNA clones were arrayed onto a Hybond-N membrane (Amersham Biosciences, Uppsala, Sweden) using a 96-well format dot blotter (Bio-Rad). Two cDNA

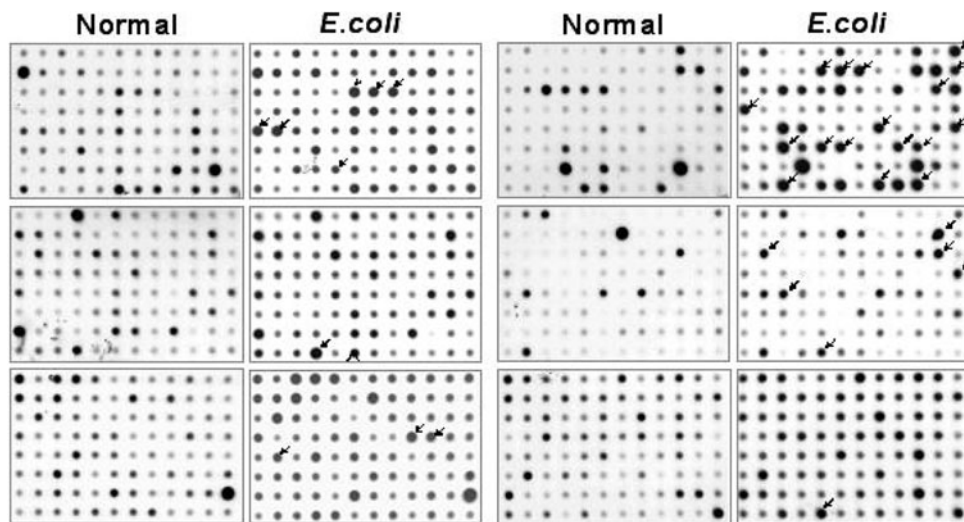


Fig. 1. Randomly selected 1,862 genes from cDNA library of *Copris tripartitus* larvae, spotted onto nylon membranes and hybridized with [ $\alpha$ - $^{32}$ P]dCTP-labelled RNA samples from normal and *E. coli* immunized larvae. Arrows indicate differently expressed clones in *E. coli* immunized larvae. Among the total of 1,862 EST clones, 13 cDNA transcripts were expressed differentially in *E. coli* immunized larvae.

probes labelled with [ $\alpha$ - $^{32}$ P]dCTP were generated by reverse transcription of 0.5–1.0  $\mu$ g poly(A)<sup>+</sup> RNA samples prepared from the normal and the immunized larvae. Probes were mixed with hybridization solution containing 50% formamide, 5  $\times$  SSC, 10  $\times$  Denhardt's solution (0.2% each of bovine serum albumin, ficoll and polyvinylpyrrolidone), 25  $\mu$ g/ml sonicated salmon sperm DNA, and 50 mM sodium phosphate (pH 7.0). Hybridization was for 3 h at 65°C. After hybridization the membranes were washed in buffers of increasing stringency, from 2  $\times$  SSC containing 0.1% SDS to 0.1  $\times$  SSC containing 0.1% SDS, 30 min in each and then exposed to X-ray film (Agfa, Wiesbaden, Germany) for 1 or 3 days at –70°C. cDNA clones selected by the dot blot analysis were sequenced using the ABI 310 Automated Sequencer (Applied Biosystems, Foster City, CA, USA). The sequences were analyzed with the BLASTX program and protein database (<http://www.ncbi.nlm.nih.gov/BLAST/>) of the National Center for Biotechnology Information (NCBI).

#### RACE (rapid amplification of cDNA ends)-PCR

The 5'-end and 3'-end RACE-PCR were performed using Marathon cDNA Amplification Kit (Clontech, Mountain View, CA, USA) according to the manufacturer's protocol. Briefly, gene-specific primers were designed from the partial cDNA sequence of the CTHSP70. These primers were used in conjunction with the anchor primer API (Clontech) to amplify the 5' and 3' ends of the DDC gene from cDNA. PCR was performed for 30 cycles of 30 s at 94°C, 30 s at 60°C, and 1 min at 72°C, followed by a 5 min final extension step at 72°C. PCR products were isolated and subcloned into the pGEM T Easy Vector (Promega) and sequenced by the dideoxy-mediated chain termination method using an ABI 310 Automated Sequencer.

#### Northern Blot Analysis

Total RNA was extracted with a Trizol reagent (Life technologies, Inc., Gaithersburg, MD, USA) from the whole larvae 0, 4, 8, 16, and 24 h after *E. coli* injection and quantified by ultraviolet spectroscopy. Aliquots of 10  $\mu$ g total RNA were fractionated on 1% agarose/6.7% formaldehyde gels and blotted onto nylon membranes (Schleicher & Schwell BioScience, Sanford, ME, USA) using 20  $\times$  SSC buffer. Membranes were hybridized with cDNA probes labelled with [ $\alpha$ - $^{32}$ P]dCTP using

a random primer labelling kit (Stratagene, La Jolla, CA, USA). Hybridization was performed for 3 h at 65°C in the ExpressHyb hybridization solution, according to the manufacturer's instruction (Clontech). Membranes were subsequently exposed to Kodak BIOMAX film (Eastman Kodak Co., Rochester, NY, USA) at 70°C. As an internal marker, 28S rRNA was visualized by ethidium bromide staining.

#### RESULTS AND DISCUSSION

As an initial step in the search for antibacterial peptides in *Copris tripartitus*, a full-length cDNA library was constructed from whole larvae of *C. tripartitus* immunized with *E. coli*. The cDNA library was plated out on LB-agarose plates covering an estimated 100,000 clones expressed in the library, and then 1,862 randomly selected cDNA clones were selected randomly from the cDNA library. Amplified PCR products were prepared and blotted onto duplicate nylon membranes. Screening was performed by differential hybridization with two types of [ $\alpha$ - $^{32}$ P]dCTP-labeled total cDNA probes synthesized from the normal and the immunized larvae.

Differential expression profiles of 1,862 clones (Fig. 1) revealed 13 cDNAs that were expressed preferentially or exclusively in the immunized larvae. Sequences of these cDNAs were determined by single-run partial sequencing and compared with the data in public databases. Seven cDNAs represented well known genes of the immune response: defensin, heat shock protein 70, peptidoglycan recognition protein, actin superfamily AT14584P, calreticulin, catalase, and transferrin. Each of these differentially expressed genes exhibited a significant protein sequence identity to the corresponding genes known from insects (Table 1). The remaining 6 cDNAs were functionally unidentified.

Insect defensins are generally cationic peptides composed of 34–46 residues with molecular mass ranging from 2–6 kDa. Initially discovered in Diptera (Matsuyama & Natori, 1988), defensins have since been described in various insects of the Neoptera cohort. Most of them act against a broad spectrum of Gram-positive bacteria and have little effect on Gram-negative bacteria and fungi (Dimarcq et al., 1994). HSP70 can be induced by various chemicals and biological stresses such as heat shock, oxidative stress, heavy metals, and amino acid ana-

1 GTTATTTCGAGCACATACCTTCGAACGGTGAGGACGTACCGCAACATTTTCGTGTCCGATTTTTTTCTAGAAATTTCCACGTTTTTACACA  
 91 CACAAGAAAGAG**ATGG**CCAAGGCTCCGGCGGTTCGATTCGATTGGGCACGACGTATTCGTGCGTCGGAGTTTTCCAACATGGAAAGGTC  
 26 M A K A P A V G I D L G T T Y S C V G V F Q H G K V  
 181 GAAATTATTGTAATGACCAAGGTAACAGAAGTACGCCCTCTTACGTCGCGTTACGGACACGGAACGTCTCATCGGAGATGCCGCAAG  
 56 E I I A N D Q G N R T T P S Y V A F T D T E R L I G D A A K  
 271 AACCAAGTCGCCATGAATCCAAATAACACTATTTTCGATGCAAAAAGATTGATTGGTCGCAGATTGATGACCATGCCGTACAAGCTGAT  
 86 N Q V A M N P N N T I F D A K R L I G R R F D D H A V Q A D  
 361 ATGAAACACTGGCCCTTCGATGTCATCAACGATGGGGGCAACCTAAATTAAGTAGAATACAAAGGGGAGGTCAAAACGTTCTATCCC  
 121 M K H W P F D V I N D G G K P K I K V E Y K G E V K T F Y P  
 451 GAGGAAGTGAGTTCTATGGTTTTAACGAAATGAAAGAGACAGCTGAAGCGTACCTAGGGAAGTCCGTAGTGAACGCCGTCATTACCGTA  
 151 E E V S S M V L T K M K E T A E A Y L G K S V V N A V I T V  
 541 CCGGCCTATTTCAACGATTCAACGTCAGCGACGAAAGATGCCGGGACAATTGCGGGACTTCAGGTTTTACGTATCATCAACGAACCA  
 181 P A Y F N D S Q R Q A T K D A G T I A G L Q V L R I I N E P  
 631 ACAGCGGTGCTATTGCGTACGGTTTAGACAAAAAGGAGTGGGAGAGAGGAACGTATTGATTTTCGACTTGGGTGGTGGAACTTTTGAT  
 211 T A A A I A Y G L D K K G V G E R N V L I F D L G G G T F D  
 721 GTATCAATTTTGACCATTTGAAGATGGTATTTTCGAGGTCAAAATCGACAGCTGGCGACACACACTTGGGTGGGAAGATTTTGATAATCGC  
 241 V S I L T I E D G I F E V K S T A G D T H L G G E D F D N R  
 811 ATGGTAAACCATTTTCGTTTCAGGAATTCAGAGGAAGTACAAGAAGGATTTGACTTCTAACAACGCGCGTTGCGCGCTCTCCGAACATCT  
 271 M V N H F V Q E F K R K Y K K D L T S N K R A L R R L R T S  
 901 TGCAGAAAGAGCGAAACGTACTCTCTCATCTCCACTCAGCGAGCATCGAAATGACTCACTGTACGAGGGTATCGACTTTTACACTTCC  
 301 C E R A K R T L S S S T Q A S I E I D S L Y E G I D F Y T S  
 991 ATTACTCGTGTCTGTTTCGAAGAGTTAAATGCAGATCTATTCCGCTCCACCATGGAACCTGTGGAGAAATCTCTTCGTGATGCTAAAATG  
 331 I T R A R F E E L N A D L F R S T M E P V E K S L R D A K M  
 1081 GACAAAGCACAGATTACGATATTGTGTTGGTGGCGGATCAACCCGATCCCAAAAGTTCAAAAACCTCTTCAAGATTTCTTTAACGGA  
 361 D K A Q I H D I V L V G G S T R I P K V Q K L L Q D F F N G  
 1171 AAGGAATTGAATAAGTCCATCAACCTGATGAGGCTGTAGCTTATGGCGCTGCCGTGCAAGCGGCTATTTTACAGGTGATAAATCTGAA  
 391 K E L N K S I N P D E A V A Y G A A V Q A A I L H G D K S E  
 1261 GAGGTTTCAGGATTTGCTGCTTTTGACGCTACGCCGTTATCGTTGGGAATTGAAACCGCCGGTGGTGTGATGACGGCTCTCATCAACGG  
 421 E V Q D L L L L D A T P L S L G I E T A G G V M T A L I K R  
 1351 AATACTACGATCCCTACGAAACAGACGACAGCTTCAACAATATTCTGATAACCAACCTGGAGTGCTTATACAAGTTTATGAAGGCGAA  
 451 N T T I P T K Q T Q T F T T Y S D N Q P G V L I Q V Y E G E  
 1441 AGAGCTATGACAAAAGATAATAACCTTTTGGGAAAATTCGAACCTACGGGAATTCACCAGCACCGAGAGGTGTCCCTCAAATTGAAGTA  
 481 R A M T K D N N L L G K F E L T G I P P A P R G V P Q I E V  
 1531 ACCTTTGACATTGACGCCAACGGTATCCTGAACGTAACGCCATTGAAAAATCCACTAACAAAGAAAAACAAATTACCATCACCAACGAC  
 511 T F D I D A N G I L N V T A I E K S T N K E N K I T I T N D  
 1621 AAGGGACGTCTCAGCAAGGAAGAAATGAACGTATGGTTAATGATGCTGAGAAGTACCGAAGCGAAGATGAGAAACAAAGGAACACTATT  
 541 K G R L S K E E I E R M V N D A E K Y R S E D E K Q R N T I  
 1711 ACAGCAAGAAGACGCCCTTGAATCCTATTGCTTCAATATCAAGCAACTATGGAGGATGAAAAATCAAGGATAAAATCACAGAGTCCGAT  
 571 T A K N A L E S Y C F N I K A T M E D E K I K D K I T E S D  
 1801 AAAACCACCGTTATGGAAAAATGAATGAAGTTATCGCTTGGTTAGATGCAAAATCAACTGGCCGATAAAGAAGAGTACGAACACAAACAG  
 601 K T T V M E K C N E V I A W L D A N Q L A D K E E Y E H K Q  
 1891 AAAGAAGTGAAGGTGTTTGCAATCCCATCATTACGAAATCTATCAGGCCGCCGGTGGAGCTCCTCCAGGAGGTATGCCAGGTTTCCCA  
 631 K E L E G V C N P I I T K F Y Q A A G G A P P G G M P G F P  
 1981 GGTGGAGCAGGGGAGCTCCTGGAGGTCCAGCACCAGGCGCTGGAAATCTGGACCAACCATCGAGGAAGTAGAT**TAA**ATTGTTTCTGT  
 661 G G A G A A P G G P A P G A G N T G P T I E V D \*  
 2071 TTTATAACCATTCATTTTAAAAAACAGTATTATTCAGACAAACTTTTTTTTTTTAATTTAAAGCTGATTTTGAATGCCGCC  
 2161 AAAGATATTTCAATATTTCAATATTCCTTTTCATATTTCTTCTGAAATATTTCTTTTTTTGTTTGTATTTATCTGGGGTATCTGTAGT  
 2251 AGTTTGGAGATTTTTCTTGTGCGTTGCATATGAACTTTGAAAAGTCT**AATA**AAAGATACAAATTACCTG**AAAAAAAAAAAAAAAAAAAA**  
 2341 **AAAAAAAAAAAAAAAA**

Fig. 2. Nucleotide and deduced amino acid sequences of the *Cthsp70* gene isolated from *Copris tripartitus*. The predicted amino acid sequence (single-letter abbreviation) is shown below the nucleotide within the open reading frame. Codons for initiation, termination, polyadenylation, and poly(A) tail are in bold. The asterisk denotes a stop codon.

TABLE 1. Differentially expressed genes in *E. coli* immunized larvae.

Representative clones	Putative identification	Species	Gene bank no.	Identity (%)	Score	E-value
SC1	NI					
SC2	Defensin-A	<i>Anomala cuprea</i>	BAD77966.1	62	107	1.00E-22
SC3	Heat shock protein 70	<i>Locusta migratoria</i>	AAP57537.3	92	317	3.00E-85
SC6	NI					
SC2-5	NI					
SC3-5	Peptidoglycan recognition SC2	<i>Drosophila melanogaster</i>	CAD89185.1	45	161	2.00E-38
SC8-5	NI					
SC10-5	AT14584p	<i>Drosophila melanogaster</i>	AAV37037.1	95	244	1.00E-63
SC11-5	Calreticulin	<i>Bombyx mori</i>	AAP50845.1	77	196	6.00E-49
SC14-5	NI					
SC29-5	Catalase	<i>Apis mellifera ligustica</i>	AAN76688.1	72	220	2.00E-56
SC31-5	Transferrin	<i>Apriona germari</i>	AAW70172.1	69	126	3.00E-28
SC33-5	NI					

NI – not identified.

logues, inhibitors of energy for energy metabolism, UV and irradiation, viral and bacterial infections, parasitism, alcohol, crowding, and inflammation (Lindquist & Craig, 1988; Hartl, 1996; Morimoto et al., 1990, 1992; Abe et al., 1995; Fukamachi et al., 1998; Wagner et al., 1999; Sierra-River et al., 1993; Hata-yama et al., 1993; Goodman & Blank, 1998; Gething & Sambrook, 1992; Sørensen & Loeschcke, 2001). Peptidoglycan recognition proteins are evolutionarily conserved molecules that are structurally related to bacterial amidases. Peptidoglycans occur in the cell walls of almost all bacteria and in many insects induce strong antibacterial responses that include activation of phenoloxidase leading to melanin formation in infected wounds. Recently, peptidoglycan recognition proteins, SC1 and SC2, were reported as downregulators in the *Drosophila* immune response (Bischoff et al., 2006). In insects, calreticulin is an early stage capsulation protein involved in non-self recognition in the cellular defense reaction (Zhang et al., 2006). Catalase is one of the regulatory enzymes and facilitates the degradation of hydrogen peroxide to oxygen and water (Collins et al., 2004). Insect transferrin is considered to be an infection-inducible gene (Yoshiga et al., 1997; Thompson et al., 2003) and has been implicated in innate immunity to microbial infection by means sequestering iron.

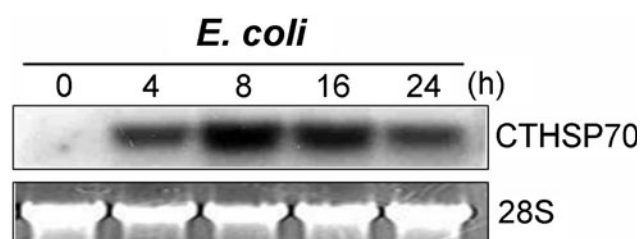


Fig. 3. Time course gene expression analysis of *Cthsp70* using Northern hybridization. *Copris tripartitus* larvae were injected with 50 µl of *E. coli* JM109 ( $5 \times 10^5$  cells) suspended in physiological saline (150 mM NaCl/5 mM KCl). Larvae were kept for 0, 4, 8, 16, and 24 h. 10 µg aliquots of total RNA were resolved on formaldehyde containing agarose gels and blotted onto nitrocellulose membranes. The probe was labelled with [ $\alpha$ - $^{32}$ P]dCTP. As internal marker, 28S rRNA was stained with ethidium bromide.

According to the aforementioned reports, HSP70 is known to be induced in response to various biological stresses including viral and bacterial infections. CTHSP70, a dung beetle homologue of HSP70, was selected for further studies because of the infection-inducible genes identified in *C. tripartitus*. HSP70 is the most likely to be a potential antimicrobial. We determined the nucleotide sequence of the CTHSP70 cDNA encoding the heat shock protein homolog of *C. tripartitus*.

In order to obtain the full cDNA sequence of CTHSP70, we performed 5'- and 3'-RACE-PCR. The full cDNA sequence of 2,354 bases contains initiation codon ATG in position 103 and the stop codon TAA at 2059 and encodes a protein of 652 amino acids (Fig. 2). Blast analysis of CTHSP70 revealed close matches with other known HSP70. The deduced amino acid sequence of CTHSP70 was highly similar to HSP70 in invertebrate and vertebrates, plants and yeasts (data not shown). The similarity was more than 70% in all matches identified from vertebrates and invertebrates.

Northern blot analysis was performed to verify differential expression of the *Cthsp70* gene in the *E. coli*-immunized larvae (Fig. 3). The expression was upregulated 4 h after *E. coli* injection, reached a maximum after 8 h and declined gradually after 16 h. These data suggest that HSP70 is involved in the immune response, which is consistent with a previous report that HSP70 expression accompanies immune response in a scallop (Song et al., 2006). Highly conserved heat shock proteins such as HSP70 are known to protect cells against stress by facilitating proper folding of nascent peptides. HSPs have been classified into several families according to their apparent mass, such as HSP90 (85–90 kDa), HSP70 (68–73 kDa), HSP60, HSP47, and low molecular mass HSPs (16–24 kDa) (Tanaka et al., 1988; Morimoto et al., 1990). Heat shock proteins are expressed in response to a wide range of abiotic stressors, but also to biological stressors such as infectious pathogens. Recent data indicate that these stress proteins have the ability to modulate the cellular immune response and play a role in protecting organisms from pathogenic stress (Encomio & Chu, 2004; Forsyth et al., 1997; Brown et al., 1993; Moseley, 2000; Iwama et al., 1998).

In conclusion, we used dot-blot hybridization to identify some pathogen-induced genes in dung beetle larvae immunized with *E. coli*. A full-length sequence of a dung beetle homolog of *hsp70* was determined and found to be up-regulated in larvae in response to infection with the pathogen *E. coli*.

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