2010-01-01

Isolation, Characterization and Molecular Cloning of DNase IIIb from Drosophila melanogaster

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By

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THESIS

Presented to the Faculty of the Graduate School of

The University of Texas at El Paso

in Partial Fulfillment

of the Requirements

for the Degree of

MASTER OF SCIENCE

Department of Biological Sciences

THE UNIVERSITY OF TEXAS AT EL PASO

May 2010

DEDICATION

Completion of this work has been long and difficult, and yet more fun and satisfying than I could have imagined before I began. There are many people that have been important parts of my life during the last two years (and some of them longer), and as such I will endeavor to thank them here. My apologies to those that I forget – rest assured that it is not intentional.

First of all, I have to thank my family. Mom – I finally made it (at least through school)! Thank you for the love and support and for pushing me to achieve. I would not have made it this far without you! I love you. To my Dad and my brother Gonzalo– the same goes for you. I don't know what I would have done without you. You have done so much for me that I can never repay you. I love you. To my love Andrew – Thanks for your support and encouragement over the years – it has meant a great deal to me.

I would like to thank Dr. Renato Aguilera for his direction, encouragement, support and friendship during my graduate studies in his laboratory. As my mentor, you have taught me much about science and the academic laboratory. As I move on to other endeavors, I am taking many things with me – two of my favorites quotes are: " Let's Rock 'N Roll", and "Work hard – play hard". Thanks Dr. Aguilera. I also have to thank the other members of the Dr. Aguilera's lab, especially Dr. Chang Soo Seong, Dr. Armando Varela, and Dr. Carolina Lema. I will always remember the "good lunch time" hours and the delicious home made Korean food. Dr. Seong – what can I say? I couldn't have asked for a better teacher and friend. I also have to thank my co-workers in the lab: Blanca Ruiz, Olivia Molinar, Lizzie Aguiniga, and Rasalin Cooper have become great friends since the day we started in the Lab.

Dr. Jeremy Ross, your encouragement and support over the last year have been tremendous! You have taught me so much in such a short time about science and protein purification. Finally, I would like to thank my friends outside the lab: Luciana Gallardo, Marisol Romero, Steven Martinez, Damaris Rosado, and Daniel Rodriguez. Thank you very much for your encouragement and support during my days of frustration.

ABSTRACT

Nucleases are enzymes that breakdown nucleic acids; they are classified by their biochemical properties into different groups (Evans *et al*., 2003). The ββα-Me finger family of nucleases are enzymes that combine structurally different groups but they are defined by a highly evolutionary conserved active site, a stretch of 22 amino acids composed by a β-hairpin (ββ) and α-helix (α) that anchor a catalytic metal ion (Sokolowska *et al*, 2009). Within this family is found the DNA/RNA non-specific nucleases. Nucleases that belong to the DNA/RNA non-specific group hydrolyze both ds and ss DNA, as well as RNA at equal or similar rates (Rangarajan and Shankar, 2001). They all share a common structure, specifically, a DNA/RNA non-specific endonuclease (NUC) domain containing a conserved Asp-Arg-Gly-His (DRGH) motif with an active site histidine (Friedhoff et al., 1994, 1996, Meiss et al., 2000).

In a recent phylogenetic study on Kamchatka crab duplex-specific nuclease (Par_DSN), a new classification of Par_DSN-like nucleases has been proposed. Conserved regions of the NUC domains from the alignment data were used for ML tree construction with TREE-PUZZLE software (Anisimova *et al.,* 2008). The NUC domains examined were divided into two main subgroups, the first containing SFN members and two insect enzymes, and the second (that they called duplex-specific nucleases, DS_NUCs) consisting of other insect and Crustacean nucleases (Anisimova *et al.,* 2008). Among this last group there are two nucleases that were uncover by our group in *Drosophila melanogaster* via microarray analysis annotated as: CG33346 and CG9989.

Deoxyribonuclease II (DNase II) is an endonuclease with optimal activity at low pH. It is localized within the lysosomes of higher eukaryotes for degradation of DNA after phagocytosis. We have previously demonstrated that down regulation of the *Drosophila melanogaster dnase II* gene, using RNA interference (RNAi) resulted in increased susceptibility to bacterial infection. In addition, genome wide expression microarray analyses of infected DNase II-deficient flies revealed over expression of a DNase-like gene that has been tentatively named *dnase IIIa*. Interestingly, a second highly homologous gene, that we have named *dnase IIIb*, was found adjacent to *dnase IIIa*.

This evidence suggests that *dnase IIIa* could be up-regulated to compensate for the lost of DNase II and function as antimicrobial response proteins. In this study we will focus on the *dnase IIIb* gene since this ORF seems to encode an authentic nuclease that can be up-regulated during bacterial infection. The putative *dnase IIIb* gene was cloned and modified to contain a GST and nine-Histidine-tag and inserted into the bacterial-expression vector pGEX-KG. DNase IIIβ was induced by addition of IPTG and the recombinant protein was then purified by GST affinity column and found to be enzymatically active. By characterizing this novel nuclease, we will be able to further determine the biological function of this enzyme during Gram-positive bacterial infection.

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INTRODUCTION

1. Nucleases

Ribonucleases (RNases) and Deoxyribonucleases (DNases) are an evolutionary conserved group of enzymes present in both prokaryotes and eukaryotes that hydrolyze phosphodiester internal bonds within double-stranded (ds) or single-stranded (ss) DNA and RNA substrates (Calvo and Ribeiro, 2006). Nucleases are key components in many biologically diverse processes such as DNA replication, repair and recombination, antiviral defense, and digestion. In addition, they play an important role in the apoptotic process (Anisimova *et. a*l, 2008). During apoptosis, dead cells are recognized and cleared by phagocytes. Phagocytes then engulf invading pathogens and nuclei expelled from erythroid precursors (Nagata, 2001). After engulfment, phagosomes further fuse with lysosomes; this results in the degradation of ingested material by nucleases within the lysosomes.

A wide variety of nucleases are known, which differ in their substrate specificities, chemical mechanisms, and biological functions (Dhananjaya, B.L., and Souza, C.J., 2010).

1.1 Beta-beta-alpha metal finger (ββα-Me-finger) superfamily of nucleases

The ββα-Me finger nucleases have been found within all biological kingdoms. These enzymes combine structurally different groups but they are defined by a highly evolutionary conserved active site, a stretch of 22 amino acids composed by a β-hairpin (ββ) and α-helix (α) that anchor a catalytic metal ion (Sokolowska *et al*, 2009). An overview of ββα-Me finger nucleases' classification is shown in figure 1.

Structurally characterized ββα-Me nucleases include homing endonucleases (e.g. I-PpoI and I-HmuI), Holliday junction resolvases (e.g. phage T4 endonuclease VII), and non-specific

nucleases [e.g. colicins, NucA, *Serratia* endonuclease, periplasmic *Vibrio vulnificus* (Vvn) endonuclease]. The ββα-Me regions of these enzymes bind one metal ion per subunit with one (e.g. I-PpoI), two (e.g. T4 endonuclease VII) or three (e.g. colicin E7) amino-acid ligands. The ββα-Me region contains a strictly conserved histidine residue that activates a water molecule for incorporation into the DNA substrate (Galburt, E.A., et al. 1999). In addition, the oligomeric state of ββα-Me endonucleases varies. Colicin E9, Vvn and the homing endonuclease I-HmuI are monomeric (Li,C.L *et al*. 2003; Shen,B.W *et al.* 2004; Pommer, A.J, *et al.* 1998). In contrast, I-PpoI and the T4 endonuclease VII form the dimers that are common for enzymes that cut both DNA strands (Flick, K.E, *et al.* 1998; Biertumpfel,C., Yang,W. and Suck,D. 2007).

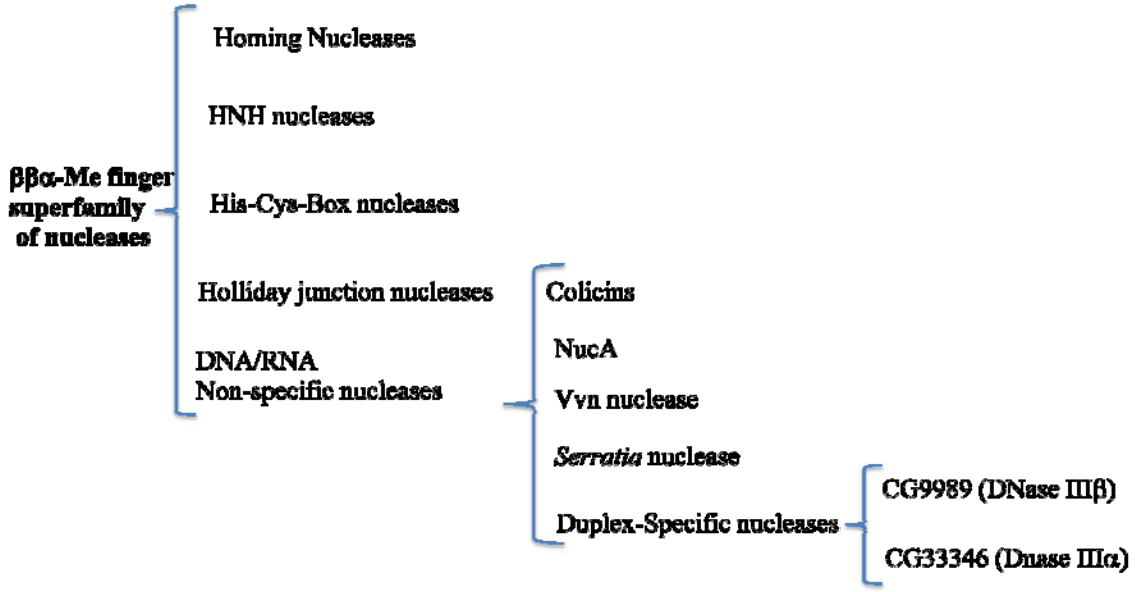

Figure 1. Nucleases classification overview

1.1.1 Homing nucleases

Homing endonucleases are enzymes that make a site-specific double-strand break at the "homing" site in intron-less alleles, thereby initiating a gene conversion event through which the intron is copied into the break site (Belfort M., *et al*., 2005). The homing endonucleases are characterized by the following sequence motifs: Leucine-alanine-glycine-leucine-isoleucine-aspartic acid-alanine-aspartic acid-glycine (LAGLIDADG), glycine-isoleucin-tyrosine-tyrosine-isoleucine-glycine (GIY-YIG), His-Cys box and histidine-asparagine-histidine (HNH). All the homing nucleases recognize lengthy asymmetric or pseudosymmetric DNA sequences (Belfort M., *et al*., 2005).

1.1.2 HNH nucleases

HNH endonucleases are characterized by a consensus sequence of ~34 amino acids that include an absolutely conserved histidine (H) that acts as a general base, asparagine (N) which function as secondary structure stabilization, and a histidine (H) that works as the metal ion ligand residue. This conserved sequence, was first reported by Shub and Gorbalenya in 1994. Mutagenesis experiments have confirmed the importance of many of these residues for catalytic activity toward DNA (Walker *et al.* 2002).

1.1.3 His-Cys box-nucleases

The His-Cys box endonucleases are part of the HNH nucleases; however, they contain an additional consensus sequences that take the form of two Cysteine (C) motifs ($CX_{2-4}C$). Some studies suggest that these Cysteines point away from the active site and coordinate structural Zn^{+} ions (Bujnicki *et al*. 2000). Examples of these nucleases are the endonuclease I-Tev III and the 5-

methylcytosine-dependent restriction endonuclease McrA (Eddy and Gold 1991; Shub *et al*. 1994).

1.1.4 Holiday junction nucleases

Homologous DNA recombination is a ubiquitous process that promotes genetic diversity and the maintenance of genome integrity in prokaryotic and eukaryotic organisms. The central intermediate in the recombination process is a four-way DNA junction usually referred to as a Holliday junction (HJ), consisting of two homologous duplex DNA molecules joined by cross-over strands (Holliday, R., 1964). Although HJ-resolving activities have been found in extracts prepared from a wide variety of organ- isms, only enzymes from bacteria, bacteriophages, yeast mitochondria, and archaea have been genetically identified and characterized. These enzymes can be divided into two functional groups. Members of the first have RuvC as their prototype, include the resolvases from bacteria, mitochondria, and archaea, have high selectivity for HJs, exhibit sequence specificity for cleavage, and are thought to have roles in recombination and DNA repair. Members of the second group, composed of the bacteriophage enzymes, cleave a variety of branched DNAs, exhibit low sequence specificity, and have roles in recombination and the processing of DNA before packaging. Absent from either group, however, is a well-characterized HJ endonuclease from metazoa or their viruses (Garcia, A. D., *et al.*, 2000)

1.1.5 DNA/RNA non-specific nucleases

In terms of biological function, these nucleases hydrolyze both ds and ss DNA, as well as RNA at equal or similar rates (Rangarajan and Shankar, 2001). In addition they do not exhibit marked base preferences, although some of them cleave substrates in a sequence-dependent and/or structure dependent manner (Cote, J., et al., 1989; Meiss, G., *et al*., 1995).

Nucleases that belong to the DNA/RNA non-specific group can be found in several bacterial strains such as *Serratia marcescens* and *Streptococcus pneumoniae*. They were also found in different microorganisms like yeast (*Saccharomyces cerevisiae*), mold (*Synephalostrum racemosum),* bovine (*Bos Taurus)*, mouse (*Mus muculus*), and parasites (*Trypanosoma brucei*). Members of this group share a common structure, specifically, a DNA/RNA non-specific endonuclease (NUC) domain containing a conserved Asp-Arg-Gly-His (DRGH) motif with an active site histidine (Friedhoff et al., 1994, 1996, Meiss et al., 2000).

1.1.5. 1 DNase colicins

The majority of the available crystal structures of the HNH-containing nucleases are of DNA-degrading bacterial toxins called colicins. These are nucleases produced by *Escherichia coli (E. coli)* during times of stress to kill competing bacterial strains. Examples are E7 and E9 colicins; their respective HNH-motif is composed of two β-strands and α-helix with a metal ion located between the structural elements.

Enzymes that contain the HNH/ββα-Me motif, and no other DNA recognition domains, as is the case for the bacterial colicins, caspase-activated DNase (CAD), and *Serratia* nuclease, show little cleavage specificity. This is consistent for the biological roles of these enzymes whose function is to degradate intracellular DNA (colicins and CAD), or, as in the case of *Serratia* nuclease, catabolize extracellular nucleic acids for nutrient uptake (Friedhoff *et al*., 1999; Widlak *et al*., 2000; James *et al*., 2002).

Several of the biochemical properties of the HNH/ββα-Me nucleases include having an optimal alkaline pH of 7.5 to 9 and requiring a single divalent cation for activity. The most

commonly used metal ion is Mg^{2+}, although many other divalent cations can substitute for it like Ca^{2+} or Mn^{2+} (Wittmayer and Raines, 1996).

The prokaryotic nucleases of this family generally contain an N-terminal signal peptide and are secreted into the extracellular environment. It is believed that nearly all of them have nutritional purposes and, possibly, also function as bactericides, similar to the colicins of *E. coli*. Because nonspecific nucleases will generally be extremely toxic to the cells that produce them, various mechanisms have evolved to deal with their toxicity. Many of the nucleases in this class contain disulfide bonds that activate the nucleases upon secretion into an oxidizing environment but when reduced in the intracellular environment, the enzyme is rendered inactive. A different nuclease inactivation system has been described in *Anabaena sp.*, where NucA is paired with a specific inhibitor, NuiA, analogous to the colicin immunity proteins in *E. coli*. NuiA protects the cell from nuclease action by forming a very stable NucA·NuiA complex (Ghosh, M., et al., 2005).

1.1.5.2 NucA

NucA, a non-specific nuclease of *Anabaena sp*., is another member of the highly active DNA/RNA non-specific nucleases. NucA is a monomeric enzyme which has a very similar secondary-structure composition to the *Serratia* nuclease. Its DNase activity is as high as that of the *Serratia* enzyme, with similar but not identical cofactor requirements, temperature, and pH dependence (Meiss, G. *et al.,* 1998).

1.1.5.3 *Vibrio vulnificus* (Vvn) nuclease

Vibrio vulnificus (Vvn) nuclease from belongs to a family of extracellular nucleases that represent another class of non-specific nucleases that are involved in protecting bacterial cells

(Li, C.L, et al., 2003). This family of enzymes includes Dns from *Vibrio cholerae* (Focareta and Manning, 1987), NucM from *Erwinia chrysanthemi* (Moulard et al., 1993), EndoI from *Escherichia coli* (Jekel and Wackernagel, 1995), and Dns and DnsH from Aeromonas hydrophila (Chang et al., 1992; Dodd and Pemberton, 1996). These endonucleases all contain a signal peptide located at the N-terminus and eight strictly conserved cysteine residues. All of these endonucleases are capable of digesting both DNA and RNA and are only active in their oxidized form (Wu et al., 2001).

1.1.5.4. *Serratia* nuclease

The *Serratia* nuclease (SmNuc) belongs to a family of DNA/RNA non-specific nucleases. A comparison of SmNuc to other endonucleases reveals differences in their targets on polynucleotides substrates. SmNuc and bovine DNase I, both cleave to yield a 3'-OH and 5'-phosphoryl whereas staphylococcal nuclease cleaves at the other side of the phosphate bond (Benedik, M. J., and Strych, U., 1998). Hence, members of this group are able to degradate both ss and ds DNA and RNA with similar efficiency and without particular sequence preferences.

Catalytically important amino acid residues of SmNuc have been identified by structural analysis, sequence comparison, and by site-directed mutagenesis. Mutations of residues R57, R87, H89, N119, and E127 resulted in enzymes that were catalytically inactive (Friedhoff, P. *et a*l., 1994; Miller, M.D, *et al*., 1994; Friedhoff, P. *et al.,* 1996).

Among the DNA/RNA non-specific nucleases are a group of Arthropod nucleases. These enzymes contain the NUC domain, however, they have substrate specificity unusual for DNA/RNA non-specific nucleases. Members of this group include the recently described duplex-specific nuclease from Kamchatka crab hepatopancreas (Shagin et al., 2002) and CuquEndo from

southern house mosquito (Calvo and Ribeiro, 2006). While CuquEndo shows no sequence specificity and it only cleaves double stranded DNA (Calvo E., Ribeiro, J.M., 2006), duplex-specific nuclease of Kamchatka crab displays a strong preference for cleaving double stranded DNA and DNA in DNA-RNA hybrid duplexes (Shagin, D.A., et al., 2002).

1.1. 5. 5 Duplex-Specific nucleases (DS_NUCs)

In a recent phylogenetic study on Kamchatka crab duplex-specific nuclease (Par_DSN), a new classification of Par_DSN-like nucleases has been proposed. Using degenerative primers, alignment software, sequence analysis with BLAST, and phylogeneric analysis, multiple sequence alignment for the Par DSN-like nucleases from several different species was obtained. Using this data a maximum likelihood (ML) tree was developed that showed a divergent evolution occurring at some point in the evolutionary past. Conserved regions of the NUC domains from the alignment data were used for ML tree construction with TREE-PUZZLE software (Anisimova *et al.,* 2008). The NUC domains examined were divided into two main subgroups, the first containing SFN members and two insect enzymes, and the second (that they called duplex-specific nucleases, DS_NUCs) consisting of other insect and Crustacean nucleases (Anisimova *et al.,* 2008). Among this last group there are two nucleases that were uncover by our group in *Drosophila melanogaster* via microarray analysis annotated as: CG33346 and CG9989 (Figure 2).

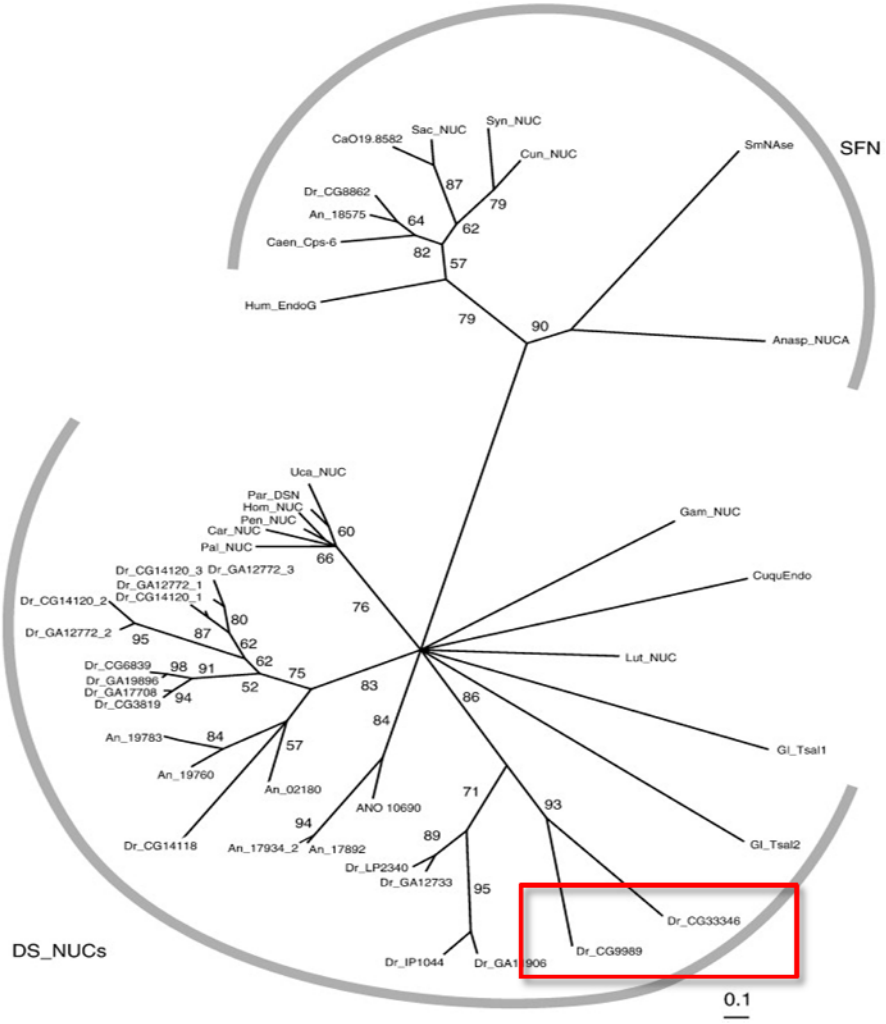

Figure 2. Unrooted maximum likelihood (ML) tree. The ML tree was constructed with TREE-PUZZLE software, based on multiple alignments of mature non-specific nucleases (Anisimova et. al, 2008). Nuclease noted in red is our nucleases of interest.

2. DNase II

DNase II is a lysosomal enzyme that works at acidic pH between 4.0-6.0 (Lyon *et. al*, 2000; Evans and Aguilera, 2003). DNase II is required in phagocytes to degrade DNA from ingested bacteria, apoptotic cells, and nuclei during erythropoiesis; the process in which red blood cells become enucleated (Lyon *et. al,* 2000).

DNase II gene was first cloned from humans and mice (Baker et al., 1998), even though the DNase II protein had already been characterized at the biochemical level as an enzyme that cleaves DNA in a ds fashion (Bernardi, 1971). DNase II homologs were later found across a wide range of species. Initial studies in the nematode *Caenorhabditis elegans (C.elegans*) demonstrated that DNase II enzymes were a key component in the degradation of DNA within phagolysosomes (Sulston, 1976). When the DNase II homologue gene (NUC-1) in *C.elegans* was mutated, persistent dead-cell nuclei within neighboring phagocytic cells as well as accumulation of DNA within the gut and ovaries was observed (Hedgecock, 1983). In mice, the targeted mutation of the DNase II gene resulted in fatal consequences to the embryos, apparently due to the lack of definitive erythropoiesis, which results in lethal anemia (Nagata, 2007). The Nuc-1 gene was originally cloned by the Aguilera's group (Evans, C.J., *et al*., 2003) by its homology with the mammalian enzyme.

Database queries performed in our laboratory identified a homologous gene (CG7780) in *Drosophila melanogaster* (Evans, Merriam, and Aguilera, 2002). In order to analyze the effect of the DNase II deficiency on the immune function in Drosophila, flies expressing *dnase II*-RNAi, which were deficient in DNase II, were examined for increased susceptibility to bacterial infection. A 70% reduction in fly viability was observed after infection with Gram-negative bacteria (*Escherichia coli)*, whereas infection with Gram-positive bacteria (*Micrococcus luteus*) reduced fly viability by 65% (Seong et al., 2006). These results suggest that immune function in *Drosophila* is weakened by DNase II down-regulation, presumably, at least in part due, to the loss of phagocytic function.

3. CG9989 (DNase IIIα) and CG33346 (DNase IIIβ) nucleases

In addition to the above-mentioned data, genome wide expression microarrays were conducted to observe the change in gene expression. Microarray data analysis revealed the over-expression of one DNase-like gene CG33346, probably as a compensation of DNase II deficiency, which we have named DNase lllα. Interestingly, a second highly homologous DNase III-like gene CG9989 was found localized adjacent to CG33346. Although the CG9989 gene was not over-expressed in infected DNase II-deficient flies, this gene caught our attention because the amino acid sequence alignment of CG9989 and CG33346 genes showed a 35% homology between both sequences. Due to this homology we named CG9989 as DNase IIIβ. Based on their proximity and peptide sequence similarity, these genes appear to have arisen by gene duplication.

1.1.5.5 The CG9989 (DNase IIIβ) nuclease

Several DNase IIIβ peptides have been detected in Schneider S2 cell extracts as reported in the Drosophila Peptide Atlas (www.mop.uzh.ch/peptideatlas/cgi/PeptideAtlas/main.cgi). This indicates that DNase IIIβ is expressed in this cell line and we confirmed this finding by quantitative Real Time PCR (qRT-PCR) analysis using Schneider S2 RNA. Furthermore, the CG9989 gene is listed in the Drosophila peptide atlas to be expressed in the larval fat body in *Drosophila melanogaster* (www.mop.uzh.ch/peptideatlas/cgi/PeptideAtlas/main.cgi). The larval fat body is in charge of producing antimicrobial peptides in *Drosophila*. This evidence suggests that the expression of the CG9989 gene in the fat body is related to a possible antimicrobial response function.

Previous microarray analyses have shown the up-regulation of the CG9989 gene (DNase IIIβ, by parasitic wasp invasion and bacterial infection with a mix of Gram positive and negative bacteria. In our lab, Oligonucleotide DNA microarrays were used for a genome-wide analysis of wild type and DNase II-deficient *Drosophila* infected with Gram-positive *Micrococcus luteus.* The CG9989 gene was found to be significantly up-regulated in infected wild type *Drosophila* in comparison to the respective non-infected control group (IC=6.7; p-value= 0.004) by 2 way ANOVA analysis (p-value cutoff= 0.05). Furthermore, when an unpaired T-test (p-value cutoff= 0.05) was applied for pair-wise comparison among infected and non-infected wild type *Drosophila*, the CG9989 gene was significantly up-regulated after infection (IC=6.6; p-value $1.94X10^{-4}$) (Unpublished data).

Hypothesis

Based on the information provided in the previous section, we hypothesize that DNase IIIβ is a novel nuclease enzyme that is involved in the immune response of *Drosophila melanogaster.*

MATERIALS AND METHODS

1. Characterization of the CG9989 (DNase IIIβ) gene

1.1 Obtaining the CG9989 (DNase IIIβ) cDNA and creating the expression clone

1.1.1 CG9989 recombinant plasmid DNA preparation

A clone of the CG9989 (DNase IIIβ) cDNA in pOT2 vector was purchased from the Drosophila Genomics Resource Center (DGRC, Indiana University, USA). Recovery of the CG9989 cDNA from FTA® (Flinders Technical Associates) clone disc was made according to manufacture's instructions: Fifty microliters (µl) of sterile 1X TE buffer were added to a microcentrifuge tube containing the Whatman® FTA® clone disc (Whatman, Inc., Florham Park, NJ). After about two seconds the buffer was discarded and the tube was placed on ice. Subsequently, 30 µl of the MAX Efficiency DH5a™ *E.coli* Competent Cells (Invitrogen, Carlsbad, CA) were added to the tube. The sample was then incubated on ice for 30 minutes, and heat-shocked for two minutes in a 37 °C water bath. The cells were transferred to a tube containing one mL of Lysogeny Broth (LB), and incubated for one hour at 37 °C in a shaking incubator (200 rpm). The transformation product (100 µl) was plated on LB agar plates containing chloramphenicol [34 µg/ml] and incubated overnight at 37 °C. The resulting colonies were grown overnight individually at 37 °C in five ml of LB medium with chloramphenicol [34 µg/ml] in a shaking incubator (200 rpm). Finally, pOT2 vector-recombinant plasmid DNA was isolated using the Mini-Prep Plasmid Isolation Kit (QIAGEN, Valencia, CA), according to the manufacturer's instructions.

1.1.2 CG9989 (DNase IIIβ) PCR amplification

Specific primers for PCR amplification of the CG9989 (DNase IIIb) gene were designed based on the genome sequence of *D. Melanogaster* (Table 1) and using Sigma-Genosys software (www.sigmaaldrich.com). pOT2 vector-recombinant plasmid DNA was used as a template. PCR amplification was carried out in a total volume of 25 µl, including 100-200 ng of DNA, 10 µM of each primer, and the following components contained in the GoTaq® Green master mix (Promega Corporation, Madison, WI): 200 µM dNTPs, 1.5 mM MgCl2, and 1 U GoTaq® DNA polymerase. The following PCR conditions were used: 35 cycles of 30 seconds at 95 °C, 30 seconds at the annealing temperature 52 °C and 1.5 minutes at 72 °C, with a final extension cycle of 7 min at 72 °C. PCR products were gel-purified using the QIA quick gel extraction kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions.

Table 1. CG9989 (DNase IIIβ) primers. Specific primers were designed to clone CG9989 (DNase IIIβ) gene initially into pGEM-T Easy vector, and later into pGEX-KG vector. A $(His)_9$ tag was introduced at the carboxy-terminus for further protein purification purposes. The forward primer contained the *EcoR I* restriction enzyme site (red bold) while the reverse primer contains the *Xho* I restriction enzyme site (blue bold) for cloning proposes. In addition, the reverse primer also includes terminal codons for the predicted CG9989(DNase IIIβ) protein.

Primer	Sequence
Forward -GST	5'**GAATTC**CGATGCCCGATCTGAAGTATATGTTGAC-3'
Reverse -$(His)_9$	5'-**CTCGAG**CTAATGGTGGTGATGGTGATGATGGCAAACACCAGTTAAGTGTGGAG-3'

1.1.3 CG9989 (DNase IIIβ) subcloning into the pGEM-T Easy vector

The CG9989 PCR product was sub-cloned into the pGEM-T Easy Vector (Promega Corporation, Madison, WI). pGEM®-T Easy Vector contains T7 and SP6 RNA polymerase

promoters flanking a multiple cloning region within the α-peptide coding region of the enzyme β-galactosidase. Inserting the CG9989 gene within the α-peptide coding region will allow identification of CG9989-recombinants colonies by blue/white screening on indicator plates containing X-gal. The ligation reaction was carried out in a total volume of 10 µl, including 25 ng of amplified PCR product producing a 3:1 insert: vector molar ratio, 50 ng of the pGEM-T Easy Vector, one µl of [10X] Rapid Ligation Buffer, and 4 U of T4 DNA ligase. The resulting pGEM-T Easy-CG9989 ligation reaction was incubated overnight (~16 hours) at 4°C.

1.1.4 Transformation of bacterial competent cells using the pGEM-T Easy-CG9989 recombinant plasmid

The transformation reaction included 50 µl of the MAX Efficiency DH5α™ *E.coli* [Genotype F- φ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rk-, mk+) phoA supE44 λ- thi-1 gyrA96 relA1] Competent Cells (Invitrogen, Carlsbad, CA) and 2 µl (100 ng of DNA) of the pGEM-T Easy-CG9989 ligation product. Following incubation on ice for 30 minutes, the samples were heat-shocked for 45 seconds in a 42°C water bath, then transferred to ice for two minutes, added one mL of Super Optimal Broth with Catabolite repression (SOC) medium, and incubated for one hour at 37 °C in a shaking incubator (200 rpm). The transformation product (100 µl) was plated on LB agar plates containing Ampicillin [50 µg/ml], 200 µl of IPTG [0.1M], and 50 µl of X-Gal [20 mg/ml] and incubated overnight at 37 °C. Positive white colonies containing the insert were screened by single-colony PCR (same protocol applied for PCR amplification of pOT2 vector-recombinant plasmid DNA) and only colonies showing the correct amplified product were grown overnight in an incubator-shaker at 37 °C in 5 ml LB medium with Ampicillin [50 ug/ml]. Finally, pGEM-T Easy- CG9989 recombinant

plasmid DNA was isolated using the Mini-Prep Plasmid Isolation Kit (QIAGEN, Maryland, USA), according to manufacturer's instructions.

1.2 DNA Sequencing

The SequiTherm EXCEL II DNA sequencing Kit-LC (Epicentre Technologies, Madison, WI) was used to confirm the correct nucleotide sequence. Two different reactions were prepared each including a different set of primers: T7 or SP6. The reaction volume of 17 µl included 135 ng of pGEM- T Easy-CG9989 recombinant plasmid DNA, 3 pmol of the primers T7 or SP6, 3.3 µl of dd-Water, 7.2 µl of [3.5X] Sequencing buffer, and 5 U (Unit) of SequiTherm EXCEL II DNA polymerase. Each reaction mixture (4 µl) was added to individual PCR tubes containing 2 µl of a different SequiTherm EXCEL II-LC termination mixes: ddATP, ddCTP, ddGTP, and ddTTP. The reaction parameters for T7 primers were 95 °C for 1 min followed by 30 cycles of as follows: 30 s at 95 °C, 15 s at 51.3 °C, and 1 min at 70 °C. The reaction parameters for SP6 primers were 95 °C for 1 min followed by 30 cycles of 30 s at 95 °C, 15 s at 44.4 °C, and 1 min at 70 °C. Once the cycles were completed, 2.5 µl of stop/loading buffer was added and the samples were incubated at 95 °C for 3 min. The reactions were sequenced using the LI-COR Long ReadIR 4200 DNA Sequencer (LI-COR, Lincoln, NE). Sequencing results were assembled using the LI-COR e-Seq V2.0 (LI-COR, Lincon,NE) DNA sequencing and analysis software. The inverted and standard sequences were aligned using ClustalW2 (www.ebi.ac.uk/Tools/clustalw2/index.html) and a CG9989 sequence (www.flybase.org).

2. Expression of GST-His-tagged DNase IIIβ protein

2.1 CG9989 (DNase IIIβ) gene subcloning into the pGEX-KG vector

The 26-kDa GST-tag is widely used as a fusion partner in the production of recombinant proteins in *E. coli*. This large tag confers several advantages such as the possibility of single-step purification using immobilized glutathione (Smith and Johnson, 1988), as well as enhanced solubility and protection against intracellular protease cleavage. In order to sub-clone the DNA encoding the DNAse IIIb into the pGEX-KG (GE Health care, Pittsburg, PA) expression vector, which adds a N-terminal GST-tag, both vectors pGEX-KG and the pGEM T Easy-CG9989 were digested with N*coI* and X*hoI* restriction enzymes. The enzyme digestion reaction included 17 µl of pGEM-T Easy-CG9989 recombinant plasmid DNA [170 ng/µl] or 5 µl of pGEX-KG vector [50 ng/µl], 2 µl of [10X] Buffer D (Promega Corporation, Madison, WI), and 5U each of NcoI and XhoI restriction enzymes (Promega Corporation, Madison, WI). The reaction was incubated overnight (~12 hours) at 37 °C. The digested plasmid was visualized under UV light using 1% agarose gel stained with ethidium bromide and only CG9989 DNA was gel-purified using the QIAquick gel extraction kit (QIAGEN, Maryland, USA) according to the manufacturer's instructions. Subsequently, the purified CG9989 gene was subcloned into the N*coI*/X*hoI* site of the pGEX-KG digested vector. The ligation reaction was carried out in a total volume of 12 µl, including 4.8 µl of the gel-purified CG9989 DNA [17.4 ng/µl], 5 µl of the pGEX KG digested Vector [23 ng/µl], 1 µl of [10X] T4 DNA Ligase Reaction Buffer, and 1 U of T4 DNA ligase (Invitrogen, Carlsbad, CA). All the reagents were mixed and the resulting ligation reaction was incubated for 3 hours at room temperature.

2.2 *Escherichia coli (E. coli)* BL21(DE3) pLysS competent cells preparation

A glycerol cell culture stock of the *E.coli* strain BL21 (DE3) pLysS cells (Invitrogen, Carlsbad, CA) was thawed; 100 µl was added to 10 ml of LB broth with chloranphenicol [34 mg/ml] and cultured overnight at 37 °C in an incubator-shaker. This culture was reseeded at a 100-fold dilution into fresh LB broth and incubated at 37 °C further until reaching an OD_{600} of 0.4 (~3 hours). The cells were harvested by centrifugation at 3500 rpm for 10 min at 4 ° C, resuspended in 10 ml of 100 mM $CaCl_2$, and incubated on ice for 10 min. After another centrifugation step, the cell pellet was again resuspended in 100 mM $CaCl_2$ to yield the final competent cell suspension.

2.3 *E. coli* BL21(DE3) pLysS competent cells transformation

Recombinant DNase IIIβ was expressed in *E.coli* strain BL21 (DE3) pLysS. The transformation reaction included 100 µl of the BL21 (DE3) pLyss *E.coli* competent cells and 3 µl (100 ng of DNA) of the pGEX_KG-CG9989 ligation product. After incubation on ice for 30 min, the sample was heat-shocked for 90 s in a 42 °C water bath, placed on ice for 2 min, added 1 mL of SOC medium, and incubated for 1 hour at 37 °C in an incubator-shaker (225 rpm). Transformation product (100 µl) was plated on LB agar plates containing ampicillin [100 µg/ml], and chloramphenicol [34 mg/ml] and incubated overnight at 37 °C. As a negative control we use the BL21 (DE3) pLysS containing an empty pGEX-KG vector lacking the CG9989 (DNase IIIβ) insert.

Positive colonies containing the insert were screened by single-colony PCR (same protocol applied for PCR amplification of pOT2 vector and pGEM-T Easy vector recombinant plasmid DNA) and only colonies showing the correct amplified product were individually

cultured overnight at 37 °C in LB medium with ampicillin [100 µg/ml] in an incubator-shaker. Finally, pGEX_KG-CG998 recombinant plasmid DNA was isolated using the Mini-Prep Plasmid Isolation Kit (QIAGEN, Valencia, CA), according to the manufacturer's instructions.

2.4 Transformed *E. coli* BL21(DE3)pLysS cells culture

Recombinant BL21 (DE3) pLysS cells expressing DNase IIIβ and negative control BL21 (DE3) pLysS cells were cultured overnight in Terrific broth (1 L: 12 g tryptone, 24 g yeast extract, 4 ml glycerol, adjust the volume to 900 ml with distilled water; re-adjust the volume to 1L with 0.17 M KH_2PO_4 and 0.72 M K_2HPO_4) with chloramphenicol (34 mg/ml) at 37 °C in an shaking incubator. The cells were reseeded at a 100-fold dilution into fresh Terrific broth and cultured at 37 °C to an OD_{600} of 0.5. Protein expression was then induced by addition of Isopropyl-β-D-thiogalctopyranoside (IPTG) at a final concentration of 1.0 mM. Culture was incubated for 3 hours at 37 °C in a shaking incubator (200 rpm). The cells were then harvested by centrifugation (10,000 rpm, 4 °C, 30 min), finally, the cell pellet was stored at -80 C until required for lysis and protein purification.

3. Cell lysis and recombinant DNase IIIβ Purification

3.1 Cell lysis

For lysis, the cell pellet collected from a 500 mL culture was resuspended in 20 ml of GST lysis buffer [50 mM $NaHPO_4$; 500 mM NaCl; 10 mM imidazole] supplemented with protease inhibitors 1 mM phenylmethanesulfonylfluoride (PMSF)] along with 2 µl/ml of lysozyme. After 30 minutes of incubation on ice the cells where processed through complete mechanical cellular disruption, the suspension was sonicated (Autotune Series High Intensity

Ultrasonic Processor with temperature controller, SONICS & MATERIALS, INC, Newtown, CT) at 4° C, 35% Amplitude, for 7 cycles of 20 seconds on ice (each cycle of sonication was followed by a resting interval of 20 seconds on ice). Cellular debris was removed by centrifugation at 12,000 rpm for 20 min at 4 °C.

3.2 GST Purification

For single GST affinity purification of GST-DNase IIIβ-His_9 protein, the resulting pre-cleared lysate was incubated with two ml of glutathione sepaharose 4B resin (GE Health care, Piscataway, NJ) for 3 hours at 4 C with mixing. Beads were washed twice with 5 resin volumes of buffer (20 mM Tris-HCl, pH8.0; 150 mM NaCl; 0.5 mM TCEP; 1mM EDTA; 100 μM PMSF). Protein was eluted with one ml of 10 mM glutathione in GST buffer (20 mM Tris-HCl, pH8.0; 150 mM NaCl; 0.5 mM TCEP; 1mM EDTA; 100 μM PMSF). Finally, samples of the eluted tagged purified recombinant GST-DNase IIIβ-$(His)_9$ were analyzed by SDS-PAGE and Western Blot.

3.4 SDS-PAGE

One volume (40 μl) of loading sample of the protein fractions was fully denatured by boiling in sample buffer [5X] (60 mM Tris-HCl; pH 6.8; 25% glycerol; 2% SDS; 14.4 mM 2-mercaptoethanol; 0.1% bromophenol blue). Denatured samples were separated on a 10% Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS–PAGE) gel. The SDS-PAGE was run for 1.5 hours at between 100 and 150 volts and then stained with Coomassie brillant blue stain [0.025% Coomassie Brillant Blue R-250 (BIO-RAD, Hercules, CA) 10 % methanol, 10 % acetic acid and 80% water] for one hour. Protein bands were observed after gel was destained in a solution consisting of 10% methanol, 10 % acetic acid and 80 % Milli-Q water.

3.5 Western Blotting

Purified GST-DNase IIIβ-$(His)_9$ protein was fractionated by SDS-PAGE on a 10% slab gel followed by transfer onto a methanol pre-soaked PVDF membrane (0.45 μm) (Thermo Scientific, Rockford, IL) for 1 hour at 0.5 Amps, blocked overnight at 4 °C using 5% BSA/ 1X TBST (10 mM Tris-HCl, pH 7.4; 150 mM NaCl, 0.05% Tween-20) and blotted with primary antibody: 1:3000 Mouse IgG Anti-GST Antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) and washed with 1X TBST, 3 times for 10 minutes. Immune reactive proteins were visualized by incubation with donkey anti-goat IgG Horseradish Peroxidase (Santa Cruz Biotechnology Inc, Santa Cruz, CA) secondary antibody at a dilution of 1:3000 for 1 hour followed by reaction with enhanced chemiluminescence substrates (ECL) activated with 30 % hydrogen peroxide. The blots were then exposed to Blue X-Ray Film (Phoenix Research Products, Candler, NC) for one minute.

3.6 Automated Electrophoresis Analysis

The molecular weight of the DNase IIIβ and GST proteins and the relative degree of each sample's target homogeneity was determined by 10% SDS-PAGE Coomassie stained and the Experion™ Automated Microfluidic Electrophoresis station (BIO-RAD Laboratories, Richmond, CA). The Experion automated electrophoresis system utilizes LabChip microfluidic separation technology and fluorescent sample detection to perform automated analysis of multiple protein samples.

4. Determination of DNase activity

To determine the pH-dependence of DNA cleavage by DNase IIIβ we have used a buffer containing 10 mM Tris, adjusted with HCl or NaOH, respectively, from pH 5 to 9 in steps of one pH unit. All cleavage reaction mixtures contained 214.8 ng/µl double stranded (ds) circular plasmid DNA (pEGFP) and 8 µl (3.5 ng/µl) of the purified fraction in a final volume of 20 µl. The reaction mixtures were incubated for one hour at 37 °C. Lastly, samples were analyzed by electrophoresis on a TAE 1.0 % agarose gel (100 mM Tris-acetate (pH 8.0), 0.5 M EDTA) containing 0.05 µg/ml of ethidium bromide and visualized under UV light.

5. CG9989 (DNase IIIβ) expression assays after Gram Positive bacterial Infection

5.1 Cell culture

Schneider S2 cells (available as frozen stocks from the Drosophila Genomics Resource Center, St. Bloomington, IN) were propagated in Schneider's Drosophila Medium (GIBCO™ from Invitrogen, Carlsbad, CA) containing Glutamax™ media supplement (Invitrogen, Carlsbad, CA) and antibiotic/antimicotic. Cell cultures were split 1:2 with fresh medium every 3-5 days, retaining one-third of the conditioned media.

5.2 Bacterial culture

Gram-positive bacteria (*Micrococcus luteus*) were quantified by serial dilutions on LB plates and colony counts. Bacterial overnight culture was cultivated to an OD_{600} of 0.38. Two samples of 20 ml culture containing 1.20 $X10^9$ bacteria were fixed by incubating in 3.5% paraformaldehyde/1X TBS [50 mM Tris-HCl, pH 7.4 and 150 mM NaCl] for 1 hour on ice prior to extensive washing (four times) at 3000 rpm for 15 minutes at 4° C in 1X TBS.

5.3 Bacterial challenge and harvest of cells

S2 Schneider cells were grown at 27 °C in HyQ SFX-Insect media (Thermo Scientific Inc, Pittsburg, PA.) containing Glutamax™ media supplement (Invitrogen, Carlsbad, CA) and antibiotic/antimicotic for 1-2 days, until a density of approximately 1 X 10^6 cells/ml was reached. Cultures containing 1.0 X 10^7 cells were inoculated with 60 *M. luteus* per cell and incubated for intervals of 24 and 48 hours at 27 °C. After infection, the cells were harvested by centrifugation at 3000 x g for 15 minutes at 4 °C and used for quantitative real-time PCR analysis.

5.4 RNA Isolation and cDNA Synthesis

Total RNA was extracted from non-infected Schneider S2 cells, as well as from bacterial-infected Schneider S2 cells respectively using TRIzol® Reagent (Invitrogen, Carlsbad, CA). Isolated RNA was quantified and qualitatively assessed by spectrophotometric OD_{260} measurements and agarose gel electrophoresis. To remove all the genomic DNA contamination from the RNA samples, 5 μg of total RNA were treated with 4 units of Turbo™DNase I using the following thermocycler program: 37 C for 38 minutes, 10 C for 5 minutes (during which 0.6 μl of 0.5 M EDTA were added to each sample to stop the DNase I activity), and 75 C for 10 minutes. To test for genomic DNA contamination, DNase I-treated RNA was PCR analyzed using the house-keeper gene rp15a and CG9989 RT-PCR primers (table 2). PCR amplification was carried out in a total volume of 20 μl, including 10 ng of RNA, 10 μM of each primer, and the following components contained in the GoTaq® Green master mix (Promega Corporation, Madison, WI): 200 μM dNTPs, 1.5 mM MgCl2, and 1 U GoTaq® DNA polymerase. The following PCR conditions were used: 30 cycles of 10 seconds at 95 °C, 15 seconds at the

annealing temperature 61 °C and 20 seconds at 72 °C, with a final extension cycle of three minutes at 72 °C.

Table 2. RT-PCR primers. Specific primers were designed using Sigma-Genosys software (www.sigmaaldrich.com), to measure the amount of transcript produced. The house-keeper gene rp15a was used as control to normalize the RT-PCR values.

Primer	**Sequence**
House Keeper (rp15a)-Reverse	5'-GTTGGTTGCATGGTCGGTGA-3'
House Keeper (rp15a)-Forward	5'TGGACCACGAGGAGGCTAGG-3'
CG9989 (DNase IIIβ)-Reverse	5'-GAATGTGGTGCGATGAAGTC-3'
CG9989 (DNase IIIβ)-Forward	5'-TGCTTAATCGACTTGGCTCA-3'.
Drosomycin Forward	5'- GCCTGTCCGGAAGATACAAG-3'
Drosomycin Reverse	5'- ATTTAGCATCCTTCGCACCA-3'

First-strand cDNA was synthesized from 1 μg of total –DNA free-RNA, using oligo DT (15 mer) primers, according to the Titan One Tube RT-PCR System Reverse Transcription Kit (Roche Applied Science, Indianapolis, IN) manufacturer's instructions. The complete reaction mixtures were thermo-cycled at 42 °C for 60 min and 85 °C for 5 min. PCR analysis was performed under identical conditions to confirm cDNA amplification for both CG9989 (DNase IIIβ) and housekeeping RP15a gene.

5.5 Quantitative real-time PCR (qRT-PCR)

Relative quantitative real-time PCR (qRT-PCR) analysis was used to measure relative levels of transcript abundance for the CG9989 (DNase IIIβ) gene following bacterial challenges.

Before the Real-Time PCR amplification process, the following dilutions were performed to create a standard curve (figure 3).

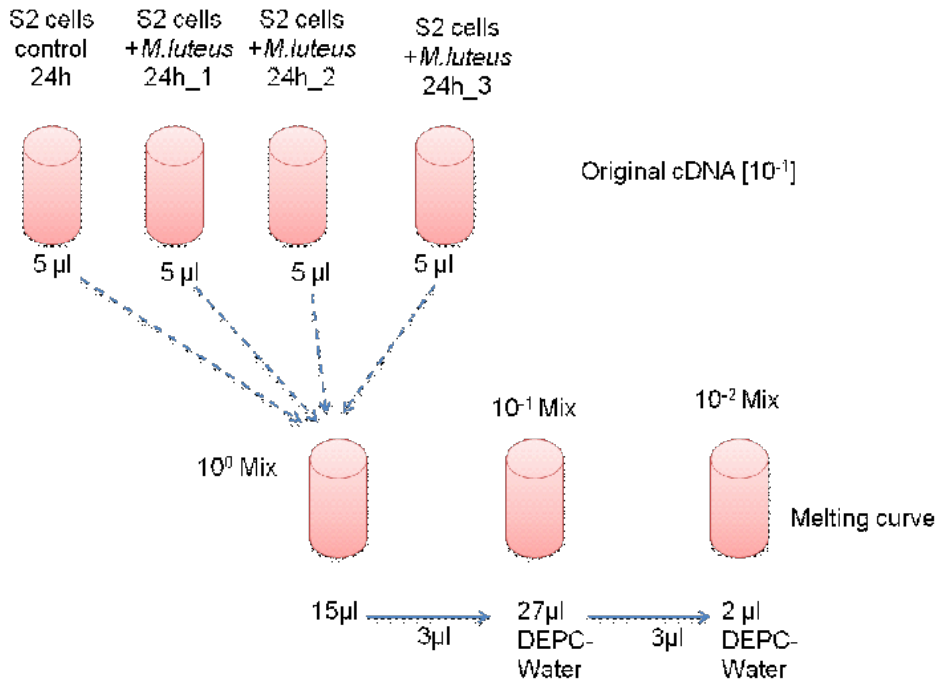

Figure 3. RT-PCR dilutions. The cDNA from different samples were 10 fold diluted and used to make the different mixtures (10^0, 10^{-1}, and 10^{-2}) that were employed during the Real-Time-PCR analysis.

Real-Time PCR amplification reactions were carried out in a total volume of 25 µl (figure 4), including one µl of sample and 24 µl of a master mix containing the following components: 12.5 µl of SYBR Green supermix (BioRad, Richmond, CA), 9.5 µl of water and one µl of each (reverse and forward) Real-Time PCR primers for the CG9989 (DNase IIIβ), Drosomycin (positive control for Gram-positive bacterial peptide induction) and the rp15a house keeper genes (table 2). The following PCR conditions were used: 40 cycles of 10 seconds at 95 °C, 30 seconds at the annealing temperature 61 °C and 20 seconds at 72 °C, with a final step of

15 seconds at 61 °C. Amplifications were performed in a Bio-Rad iCycler (Bio-Rad, Richmond, CA).

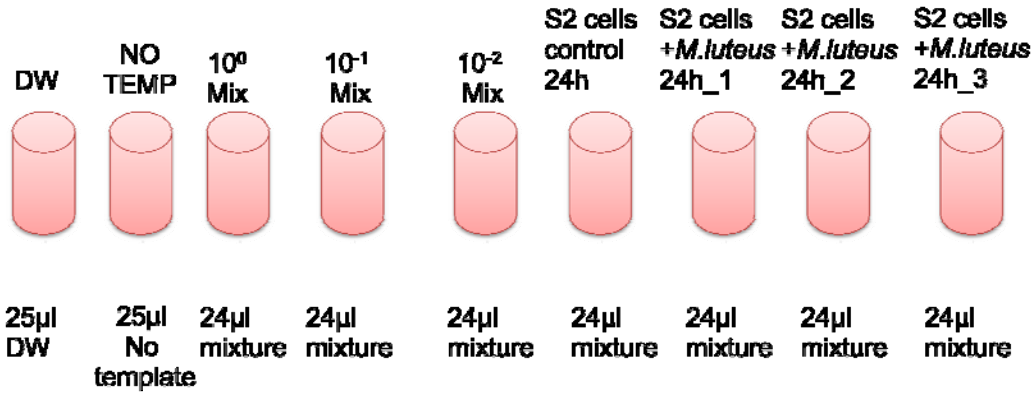

Figure 4. Real-Time PCR amplification reactions. The Real-Time amplification reactions were carried out in a 25 µl total volume per well. The iCycler iQ Real-Time PCR Detection System software analyses all the wells and calculates the average cycling threshold (Ct).

5.6 Calculations for gene expression profiles

Average cycling threshold (Ct) values, defined as the point at which the fluorescence rises above the background fluorescence, were obtained using the iCycler iQ Real-Time PCR Detection System software (Bio Rad Laboratories, Richmond, CA). The maximum Ct value was set at 20. These Ct values were recorded in Microsoft Excel 2003 for subsequent calculations. Results were analyzed using the $2^{\Delta\Delta Ct}$ method and a standard mathematical model. All data were normalized to rp15a expression levels from the same individual sample.

RESULTS

1. Characterization of the CG9989 (DNase IIIβ) gene

1.1 CG9989 (DNase IIIβ) gene PCR amplification

The gene CG9989 (DNase IIIβ) is located on the third chromosome at position 3R:98E1 (See figure 5). In order to obtain the specific cDNA sequence that codes for CG9989 (DNase IIIβ), a specific 1137 base-pair (bp) PCR product of the CG9989 (DNase IIIβ) gene was amplified from pOT2 vector that contains the CG9989 gene. Recombinant CG9989-pOT2 vector was purchased from the Drosophila Genomics Resource Center (DGRC, Indiana University, USA) The PCR product was visualized under UV light using 1.0 % agarose gel stained with 0.5 μg/ml ethidium bromide (Figure 6).

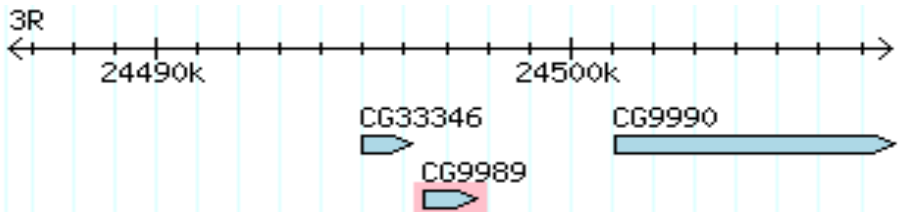

Figure 5. Position of the CG9989 gene on the third chromosome of *Drosophila melanogaster.* The sequence location is 3R:24,496,458..24,497,743 [+] (flybase.org).

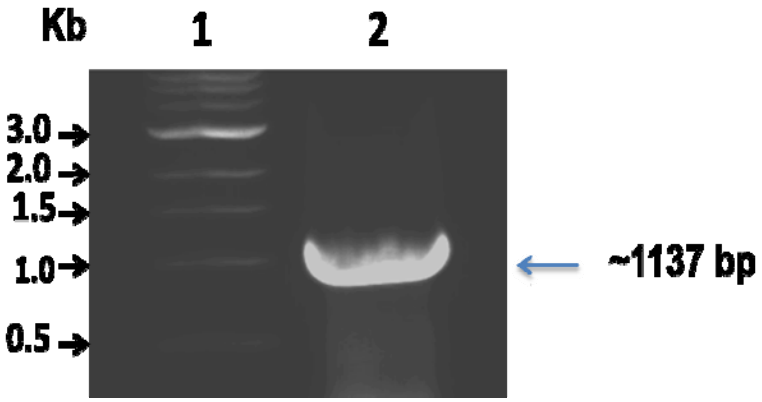

Figure 6. **CG9989 (DNase IIIb) PCR product.** PCR-amplified product was visualized using 1 % agarose gel stained with ethidium bromide, gel purified (QIAEX II, Qiagen, Valencia, CA) and cloned initially into pGEM-T Easy vector. 1. 1 Kb DNA ladder (New England BioLabs Inc, Ipswich, MA); 2. CG9989 PCR product (1137 bp).

1.2 CG9989 (DNase IIIb) subloning into pGEM T- Easy vector and transformation of bacterial competent cells

The CG9989 (DNase IIIβ) PCR product was cloned into pGEM T-Easy Vector, which was chosen because it contains Thymine (T)-overhangs at the insertion site that improve the efficiency of ligation of PCR products by preventing recircularization of the vector. The vector in addition has a blue/white selection system (X-Gal) and provides accurate and rapid ligation.

The pGEM-T Easy-CG9989 ligation product was used to transform the competent DH5α™ *E. coli* bacterial cell strain. Screening of the correct colonies was done by choosing positive white colonies and performing single-colony PCR analysis on all them. All five colonies tested showed the correct size fragment when compared with the original PCR product used as the positive control (Figure 7).

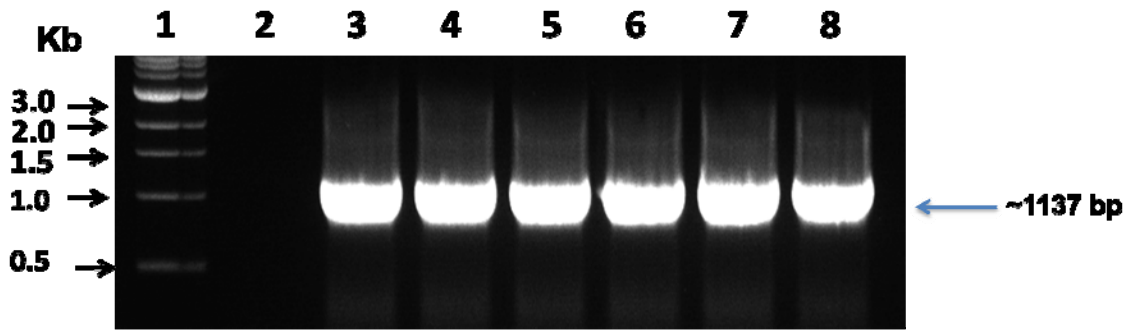

Figure 7. PCR product of CG9989 (DNase IIIβ) gene from *E.coli* DH5α™ transformed cells. Single colony PCR was performed on several clones. 1-1 Kb DNA ladder (New England BioLabs Inc, Ipswich, MA); 2-No template (negative control); 3- Original PCR product (positive control); 4,5, 6, 7, and 8- CG9989 (DNase IIIb)-pGEM-T Easy transformed clones.

1.3 DNA Sequencing of the CG9989 (DNase IIIβ) gene

Plasmid DNA from several CG9989-(DNase IIIβ)-pGEM-T-transformed bacterial clones was sequenced using T7 and SP6 primers contained in the pGEM-T Easy Vector. Of all five clones, only two clones showed perfect alignment with the CG9989 sequence from flybase (www.flybase.org, Accession No. is: ID=FBgn0039593). Alignment of DNA sequences was performed using the ClustalW2 software (www.ebi.ac.uk/Tools/clustalw2).

Analysis of the resulting CG9989 DNA sequence (without the GST tag) revealed an 1131-nucleotide open reading frame (starting with the first initiator codon) that encodes a 378-amino acid protein (Figure 8). Submitting this amino acid sequence into two different ExPasy software tools: PSORT (Prediction of protein sorting signals and localization sites in amino acid sequences, Institute for Medical Science, University of Tokyo, Japan) and SOUSI (Classification and secondary structure prediction of membrane proteins, Nagoya University, Japan), these analyses reveled a likely soluble protein that could be localized in an extracellular region, respectively. On the other hand, according to TMpred (TMpred - Prediction of Transmembrane

Regions and Orientation, ExPasy) this protein has two strong transmembrane helices (see figure 8). The relevance of this information is that it may indicate that the enzyme is localized in the membrane. In addition, BLAST searches were performed against the non-redundant protein National Center for Biotechnology Information (NCBI) database. According to BLAST, this protein sequence possesses similarities to several DNA/RNA non-specific nucleases.

M A M P D L K **Y M L T I L S L Y F F V G S V Q A N C L I** D L A H L N A N Y V Y L S Q N N G V Y D I Q
R S D I V E I H Q T L Y L L C N G G L H R T T F L C R Y D S V F S P A L S S A A C A P P D P V V V K V P
D **T S C S I P S A T F A V G F S F N G R F M E L Y** R N C F D G Y S L A F Q H S I Y K A Y R Y V N T V P
R P N P T W Q S D Q L S G G F D N A Y E G R A T Q A C L L T N L G A V Q P Q C K F D R G H M T P A
S A F I S T E L K K S T F R Y L N A I P Q Y R G V N R G K W K A V E T W V N N M V R G L Y D N P I I N
N V Q I P R T Y D V L K V C I G A L G V H R L R H N T N N N M I P I Y L L D N N K I P V P E W M Y K I
V S H L S G D K W V M L T Y N D V S L P N Q Q A L N Q I C H V I P C H P G L N L N T K D V G H T V C
C D P Y R F I T I N A P H L T G V C H H H H H H H H H

Figure 8. Amino acid sequence of DNase IIIβ protein. The CG9989 (DNase IIIβ) DNA sequence was translated using the Translate Tool software, ExPasy (www.expasy.ch/cgi-bin/dna_aa). The His-tag amino acid sequence is in gray. In bold, predicted transmembrane domains, according to TMpred - Prediction of Transmembrane Regions and Orientation software, ExPasy (www.expasy.ch).

2. Amino acid sequence analysis and 3D structure prediction

Alignment of the DNase IIIβ putative active center (Figure 9) with other DNases of the DNA/RNA non-specific nucleases family shows the presence of the conserved Arginine, Glycine, Histidine (RGH) triad found in most non-specific nucleases characterized so far. The importance of these residues for catalysis has been previously studied in detail (Miller et al., 1999; Shlyapnikov et al., 2000).

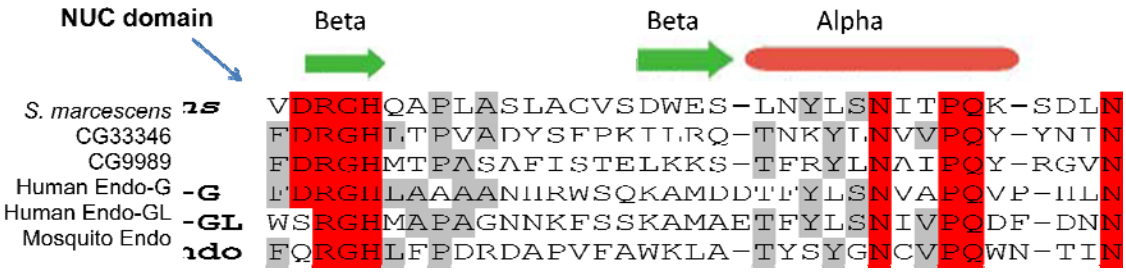

Figure 9. Amino acid sequence alignment of conserved elements among different non-specific nucleases' active sites. Conserved amino acid residues are highlighted in red and grey. Most known nucleases have the conserved RGH triad called the NUC domain.

It is also interesting to note that the active site of *SmNuc* involves several highly conserved amino acid residues: His143, Asn174, Glu182, plus the Asn119 residue ligand of the magnesium atom, which is important for catalytic activity (Ghosh, M, *et al*., 2005). These four amino acids residues are highly conserved among DNases and equidistantly localized in DNase IIIβ, forming the catalytic site for phosphodiester bond cleavage.

The hypothetical 3D structure of novel DNase IIIβ was determined by comparison with non-specific endonuclease from *Serratia marcescens*. For modeling purposes, the amino acid sequence of DNase IIIβ nuclease was submitted to the ExPASy proteomics server of the Swiss Institute of Bioinformatics (SIB) software (www.swissmodel.expasy.org/workspace). Monomer of endonuclease from *Serratia marcescens* was selected as comparison structure, based on software criteria. The software compares the amino acid sequence based on models of homologous proteins of known structure (Figure 10)**.**

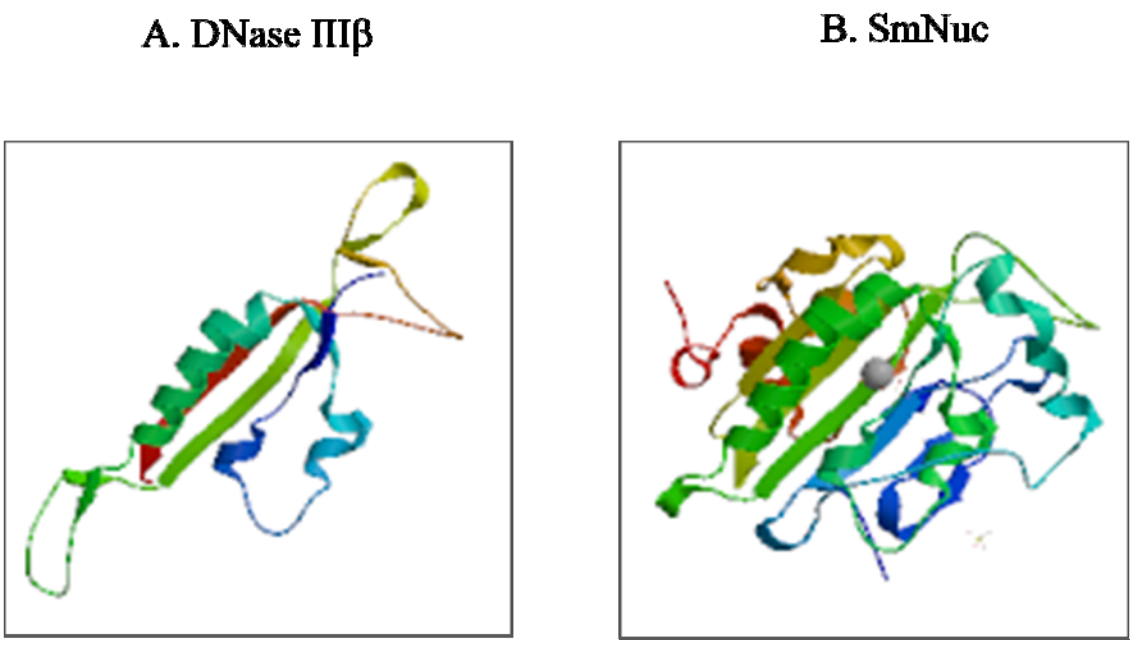

Figure 10. Hypothetical 3D structure of novel DNase IIIβ. Amino acid sequence of DNase IIIβ was submitted to the swiss model software, ExPASy (www.swissmodel.expasy.org/workspace) the 3D structure of DNase IIIβ (A) was predicted by ExPASy based on the comparison of amino acids 189-307 to *Serratia mercesens* endonuclease (B).

3. Expression of GST- DNase IIIβ–$(His)_9$ protein construct

3.1 CG9989 (DNase IIIβ) gene subcloning

The full length $(His)_9$ tagged- CG9989 (Dnase IIIβ) insert was cloned in frame into the *Eco*R I/*Xho* I site of the pGEX-KG vector to generate a GST-DNase IIIβ-$(His)_9$ fusion protein (Figure 11). The pGEX-KG vector, which is a commonly used vector to express proteins, carries an N-terminal Glutathione S-Transferase (GST) Tag sequence followed by a thrombin site.

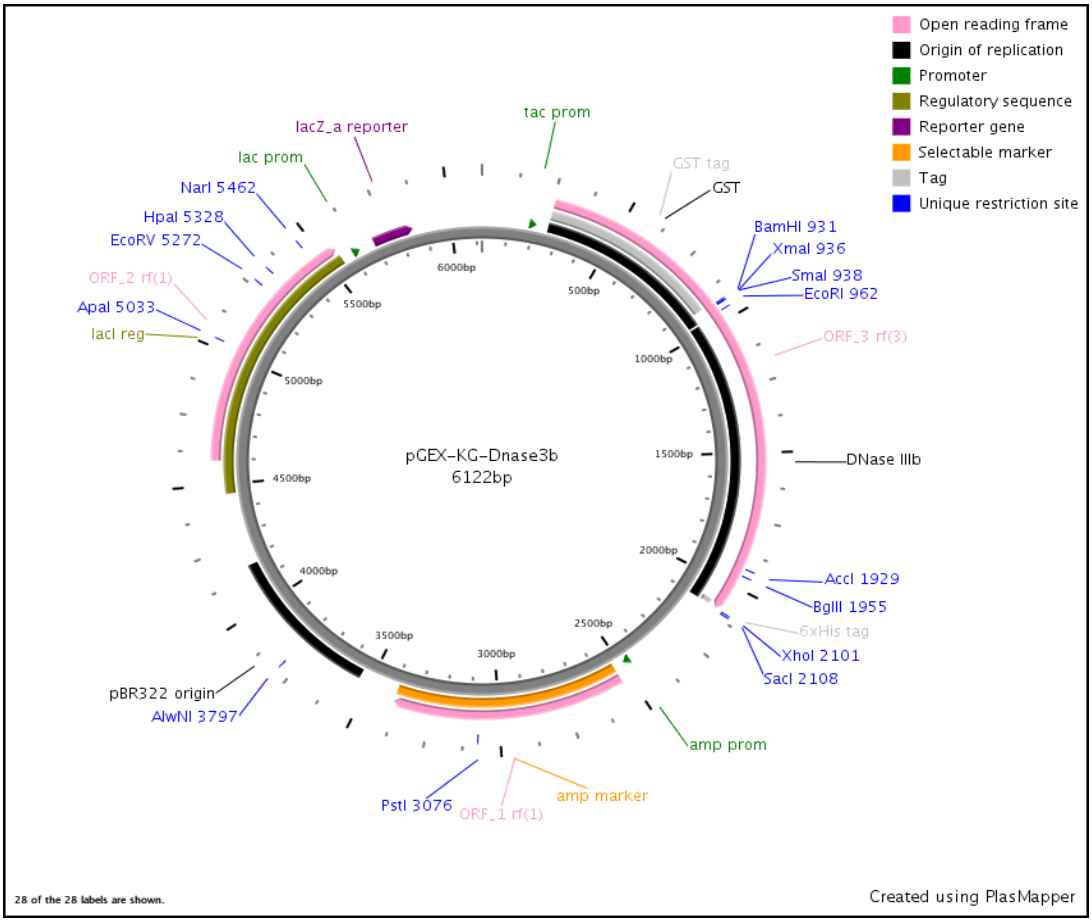

Figure 11. Map of the pGEX-KG-DNase IIIβ plasmid. A map of the pGEX-KG containing the DNase IIIβ insert was created using the PlasMapper software (http://openwetware.org/wiki/Plasmapper). The insert clone is localized between *Xho I* and *EcoR I* restriction enzyme sites.

To subclone into the pGEX-KG expression vector, the CG9989 (DNase IIIβ)-pGEM-T Easy-containing plasmid was digested with *EcoR*I and *Xho* I restriction enzymes. As a result, the CG9989 (DNase IIβ) insert was released (Figure 12) and then ligated to the pGEX-KG vector.

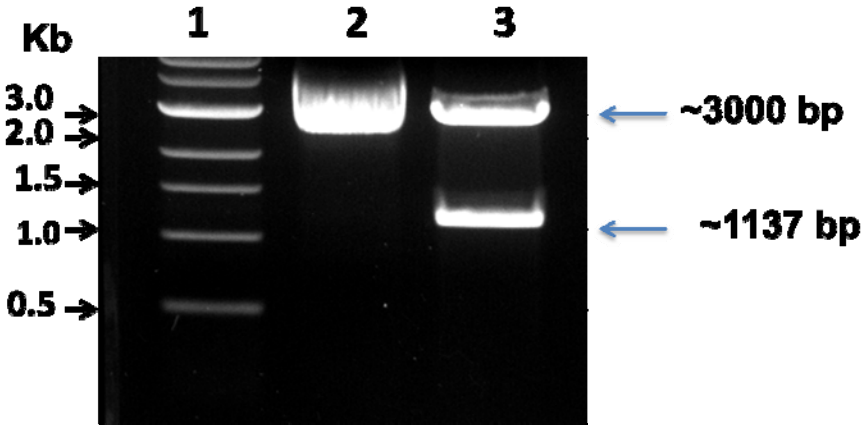

Figure 12. CG9989 (DNase IIIβ) gene subcloning. Sequenced pGEM T-Easy-CG9989 recombinant plasmid DNA was digested with *EcoR* I and *Xho I* restriction enzymes to obtain the CG9989 insert for subcloning purposes. The excised CG9989 (DNase IIIβ) fragment was gel purified and subcloned into pGEX-KG bacterial expression vector. 1. 1 Kb DNA ladder (New England BioLabs Inc, Ipswich, MA); 2. Non-digested pGEM T Easy-CG9989 recombinant plasmid; 3. *EcoR* I/X*ho* I digested pGEM T Easy-CG9989 recombinant plasmid.

The predicted GST-DNase IIIβ-$(His)_9$ recombinant protein has a molecular mass of 70.071 KDa and an isoloectric point (pI) of 7.84 according to the Protein Calculator v3.3 software (http://www.scripps.edu/~cdputnam/protcalc.html).

3.2 Recombinant GST-DNase IIIβ-$(His)_9$ protein expression

E.coli strain BL21 (DE3) pLysS cells were transformed with an aliquot of the ligation mixture. To verify if the clones contained the CG9989 (DNase IIIβ) gene, single colony PCR was performed. PCR products were visualized under UV light using 1% agarose gel stained with ethidium bromide. Only three out of four clones showed a specific PCR product of 1181 bp (Figure 13).

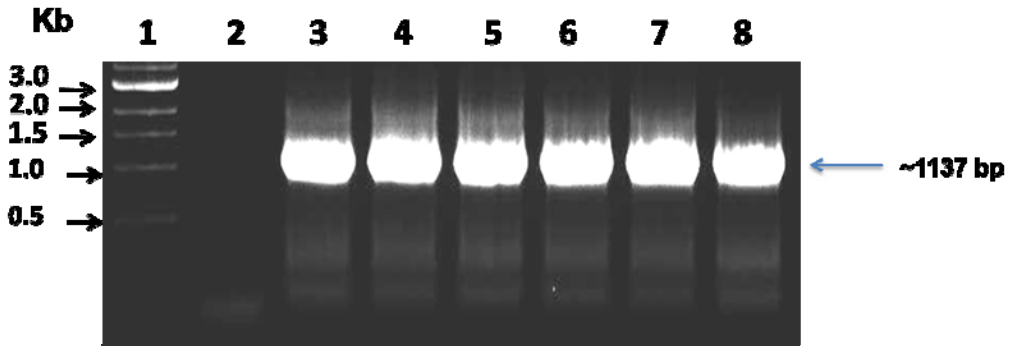

Figure 13. PCR product of CG9989 (DNase IIIb) gene from *E.coli* BL21(DE3)pLysS transformed cells. Single colony PCR was performed to several clones. 1. 1 Kb DNA ladder (New England BioLabs Inc, Ipswich, MA); 2. No template (Negative control); 3- Original PCR product (positive control); 4, 5, 6, 7, and 8. CG9989 (DNase IIIβ)-pGEX-KG transformed clones.

Recombinant GST-DNase IIIβ-$(His)_9$ protein was expressed after IPTG induction of BL21 (DE3) pLysS selected clones. After determining the optimal temperature and time intervals for IPTG induction as well as the optimal wavelength for measuring bacterial growth, our data showed that the highest yield of soluble protein was produced using a temperature of 37 C with an OD_{600} = 0.5 and an induction time of 3 hours.

In addition, western blotting analysis reveled that the expression of the ~70 KDa recombinant GST-DNase IIIβ–$(His)_9$ protein was present as a soluble protein (in the clarified supernatant) but also in the inclusion bodies (in the pellet) after bacterial cell disruption (Figure 12). Therefore, GST-DNase IIIβ-$(His)_9$ recombinant protein was isolated under native conditions which will allow us to make direct DNase assays on the active protein.

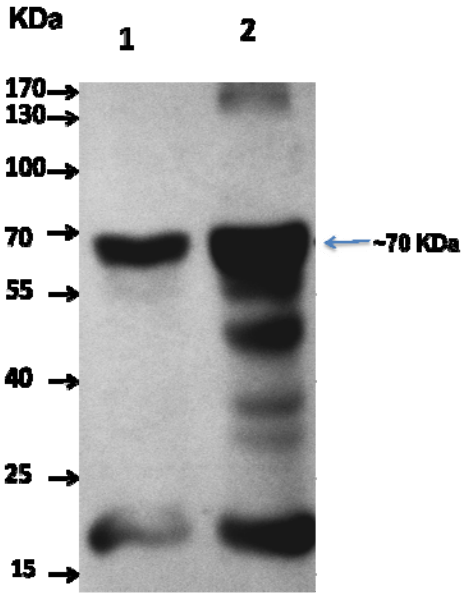

Figure 14. Expression of DNase IIIβ protein. BL21 (DE3) PlysS cells containing the pGEX-KG-DNase IIIβ expression vector were lysed and IPTG-induced for expression of the DNase IIIβ protein; the bacterial lysate was then loaded on SDS-PAGE and immunobloted with GST antibodies. 1.Transfected and IPTG-induced bacteria cells expressing GST-DNase IIIβ-$(His)_9$ protein in the supernatant fraction; 2.Transfected and IPTG-induced bacteria cells expressing GST-DNase IIIβ-$(His)_9$ protein in the pellet fraction after bacteria disruption. Arrows point to the pre-stained protein markers in Kilo Daltons (KDa).

4. Purification of GST-DNase IIIβ-$(His)_9$

GST-affinity purification method was used to isolate recombinant DNase IIIβ. All the stages of purification were followed by SDS-PAGE with Coommasie staining and GST immunoblotting. In the purification step the GST tag was used to separate the GST-tagged proteins from the total lysate. Hence, SDS-PAGE Coomassie blue staining analysis of the dual affinity purification showed two main bands at the expected molecular weight of 70 kDa with an impurity at about 26 kDa. This molecular weight of 26 kDa corresponds to that of the untagged GST purified protein. The final affinity purified fraction was also analyzed by Western blotting

and probed with GST antibody. Two bands were observed at the correct molecular weight of 70 kDa and 26 kDa (Figure 15).

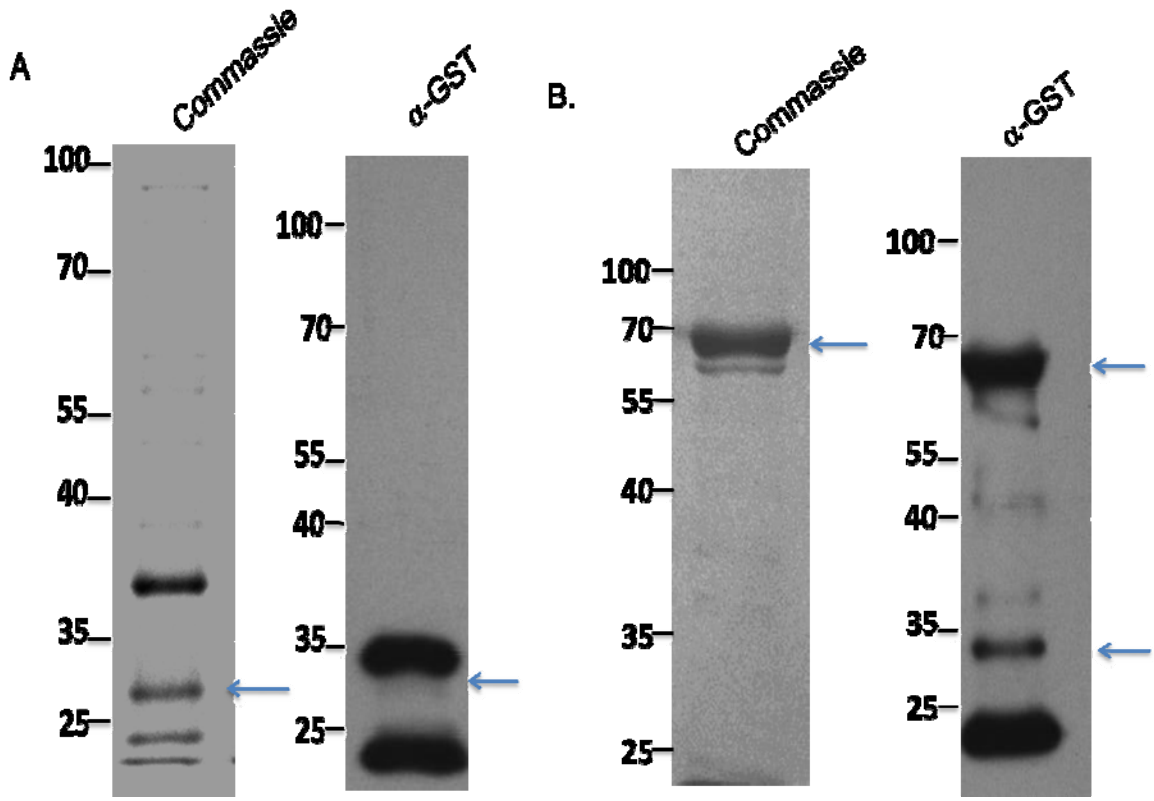

Figure 15. Lysis and affinity purification of GST and recombinant GST-DNase IIIβ-$(His)_9$. Protein samples were separated on a 10% SDS-PAGE gel and visualized after coomassie blue staining or transferred onto a nitrocellulose membrane for western blotting. For western blot analysis, the membrane was probed with anti-GST antibody. A. Commasie blue stained SDS-PAGE gel and western blotting analyses of eluate after single affinity GST tag purification of GST -produced using the pGEX-KG expression vector without the insert– B. Commasie blue stained SDS-PAGE gel and western blotting analyses of eluate after single affinity GST tag purification of recombinant GST-DNase IIIβ-$(His)_9$.

In addition, the relative degree of each sample homogeneity was determined by the Experion™ Automated Microfluidic Electrophoresis station (BIO-RAD Laboratories, Richmond, CA). Results of this analysis indicated a 60% purity of the DNase IIIβ protein (Figure 16).

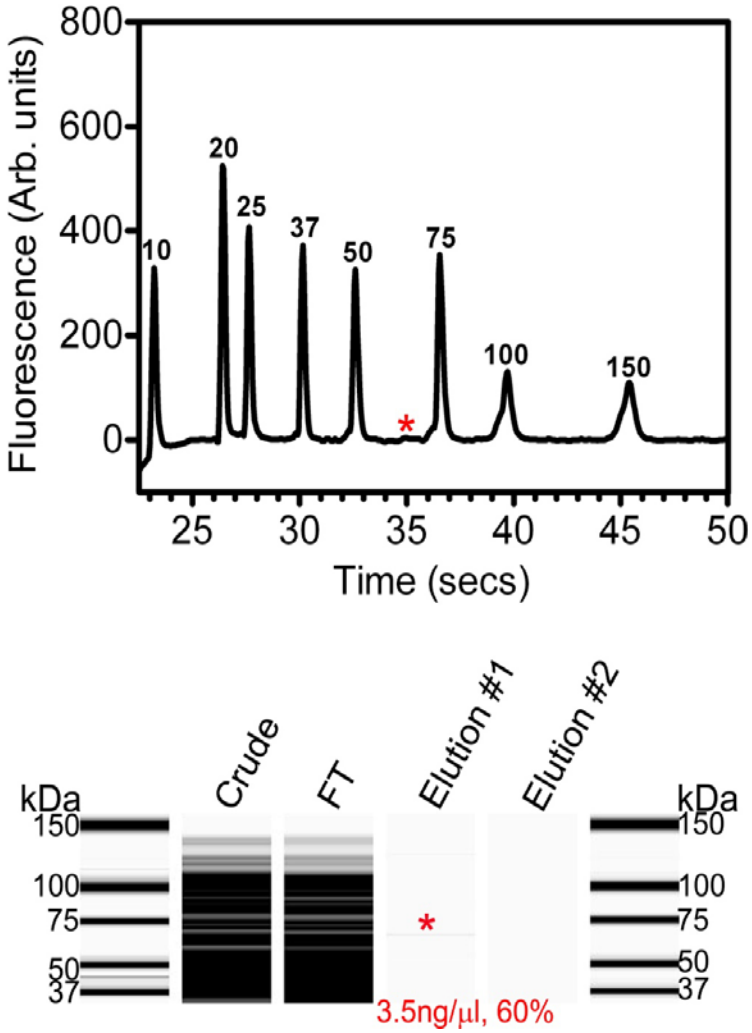

Figure 16. Sample homogeneity analyses. The purified fraction was submitted to a purity analysis using the Experion™ Automated Micro fluidic Electrophoresis station electrophoresis. The DNase IIIβ was present in the fraction as 60% pure with a concentration of 3.5 ng/µl (star).

5. DNase Activity

The purified fraction was tested for DNase activity. Recombinant DNase IIIβ activity was examined for divalent cation sensitivity. DNase IIIβ hydrolyzed 214.8 ng/µl double stranded (ds) circular plasmid DNA in the pH range 5.0-9.0 in the presence of 1 mM $MgCl_2$. Figures 17, 18, and 19 show that in the presence of Magnesium (Mg^{+2}) ions, DNase IIIβ hydrolyzes the DNA substrate into a smear of heterogeneous digested product after one, three and eight hours of incubation at 37 C. The nuclease activity of DNase IIIβ was not detected in the reaction mix depleted of DNase IIIβ (negative control). The data indicates that DNase IIIβ is divalent cation dependent, similar to *S. marcescens* endonuclease, which requires Mg^{+2} ions (Friedhoff *et al.,* 1996).

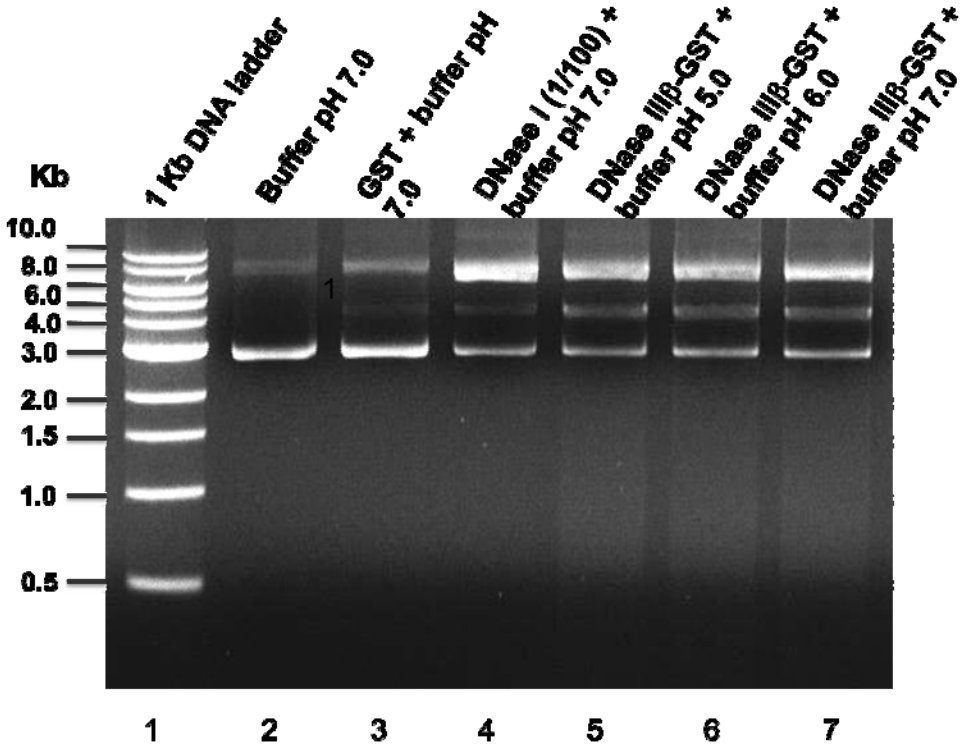

Figure 17. DNase activity of DNase IIIβ against double stranded (ds) DNA after one hour. Agarose gel electrophoresis of ds DNA after one-hour incubation at 37 C. Lines: 1, 1-Kb DNA ladder; 2, ds DNA (214.8 ng/μl) in activity buffer (1 mM $MgCl_2$, 50 mM NaCl; 20 mM Tris-HCl) at pH 7.0; 3, affinity purified GST in the presence of ds DNA (214.8 ng/μl) in activity buffer at pH 7.0; 4, commercial bovine Turbo DNase I (1:100 diluted GST) in the presence of ds DNA (214.8 ng/μl) in activity buffer at pH 7.0; 5, 6, 7,recombinant affinity purified GST-DNase IIIβ-$(His)_9$ in the presence of ds DNA (214.8 ng/μl) in activity buffer at pH 5.0, 6.0, and 7.0.

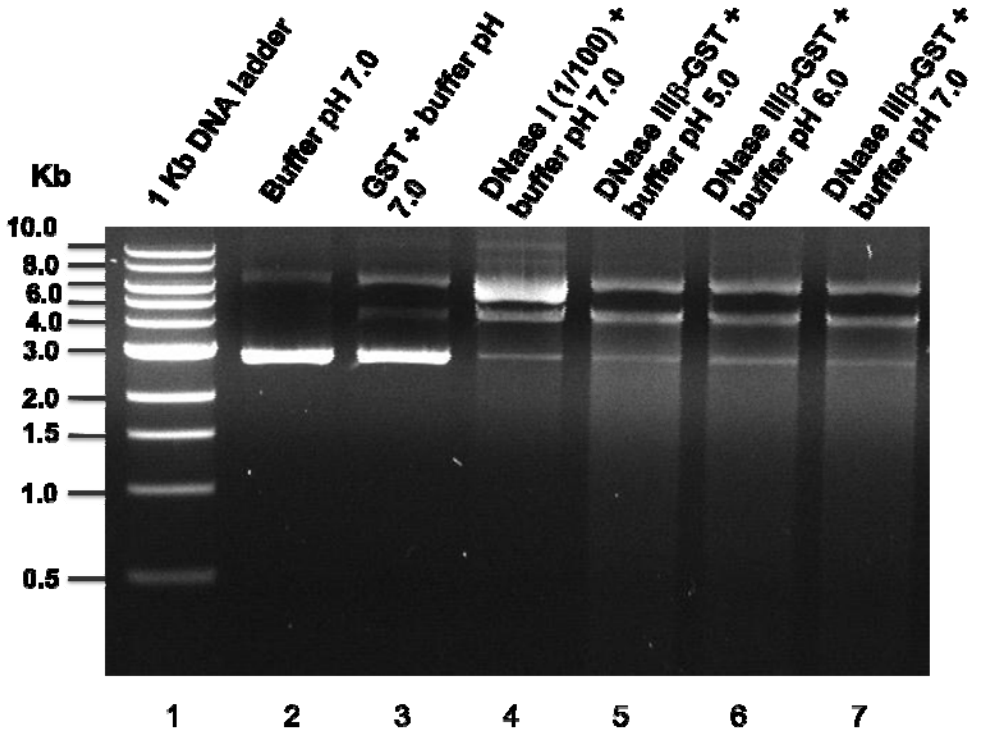

Figure 18. DNase activity of DNase IIIβ against double stranded (ds) DNA after three hours. Agarose gel electrophoresis of ds DNA after three-hour incubation at 37 C. Lines: 1, 1-Kb DNA ladder; 2, ds DNA (214.8 ng/µl) in activity buffer (1 mM $MgCl_2$, 50 mM NaCl; 20 mM Tris-HCl) at pH 7.0; 3, affinity purified GST in the presence of ds DNA (214.8 ng/µl) in activity buffer at pH 7.0; 4, commercial bovine Turbo DNase I (1:100 diluted GST) in the presence of ds DNA (214.8 ng/µl) in activity buffer at pH 7.0; 5, 6, 7, recombinant affinity purified GST-DNase IIIβ-$(His)_9$ in the presence of ds DNA (214.8 ng/µl) in activity buffer at pH 5.0, 6.0, and 7.0.

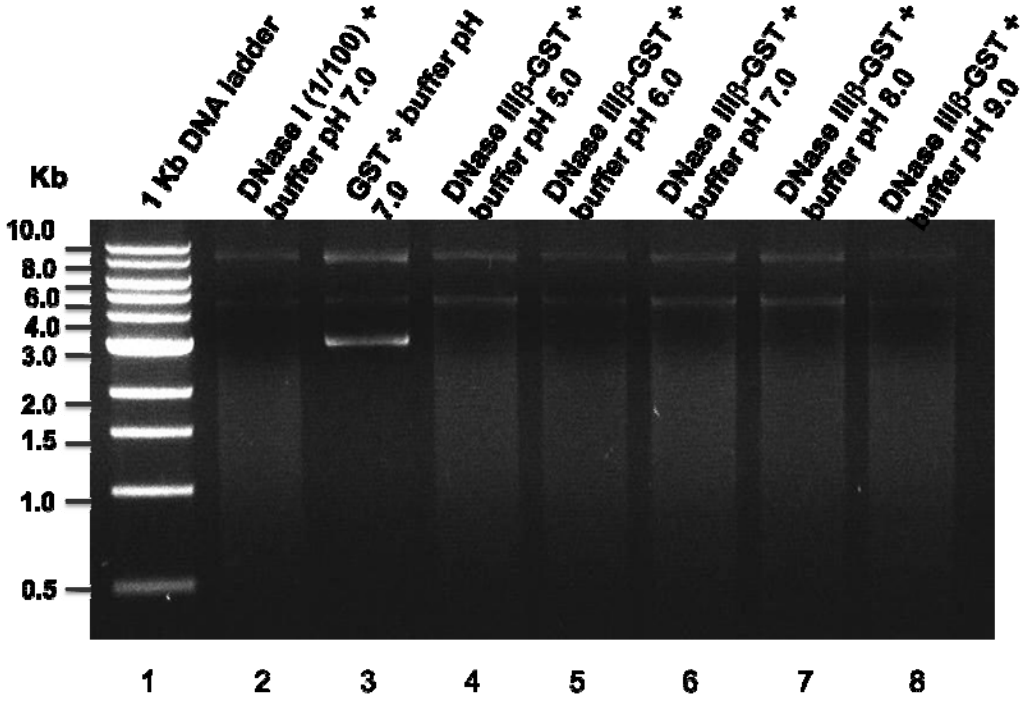

Figure 19. DNase activity of DNase IIIβ against double stranded (ds) DNA after eight hours. Agarose gel electrophoresis of ds DNA after eight-hours incubation at 37 C. Lines: 1, 1-Kb DNA ladder; 2, commercial bovine Turbo DNase I (1:100 diluted GST) in the presence of ds DNA (214.8 ng/μl) in activity buffer at pH 7.0; 3, affinity purified GST in the presence of ds DNA (214.8 ng/μl) in activity buffer at pH 7.0; 4, 5, 6, 7, and 8, recombinant affinity purified GST-DNase IIIβ-$(His)_9$ in the presence of ds DNA (214.8 ng/μl) in activity buffer at pH 5.0, 6.0, 7.0, 8.0, and 9.0.

6. RT-PCR analysis

We used quantitative RT-PCR analysis and time course experiments to compare the relative abundance of the CG9988 (DNase IIIβ) transcript in Schneider S2 cells following bacterial challenge with a Gram-positive bacteria *M. luteus*. However, the relative abundance of CG9989 transcripts was not significative increased after 24 hours infection with *M.luteus*. In the other hand, the transcript abundance in our positive control Drosomycin- an antimicrobial peptide that over expresses after *M.luteus* infection- was increased in a 3.15 fold (Figure 20).

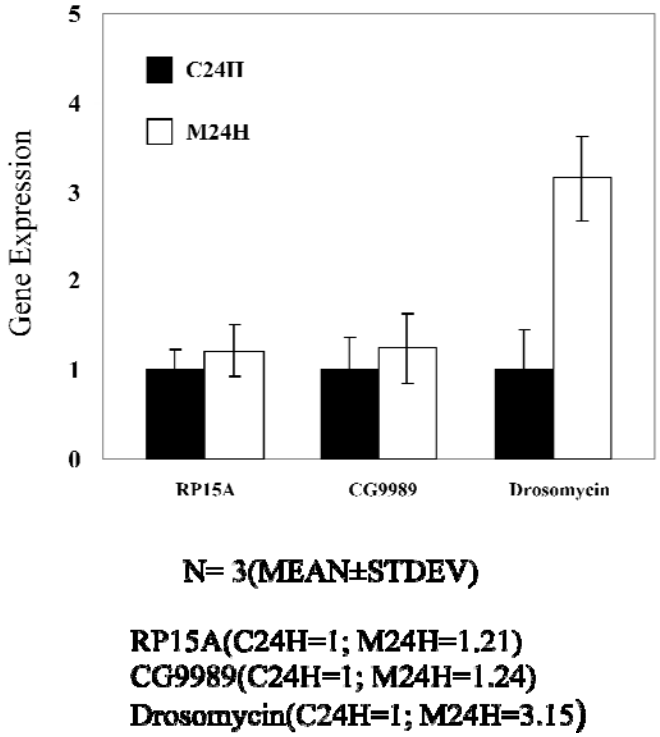

Figure 20. Quantitative real-time PCR analysis. Analyses of CG9989 (DNase IIIβ) transcript levels in Schneider S2 cells after infection with *M.luteus*. Transcripts levels for RP15A, Drosomycin, and CG9989 were measured at 24 hours post-infection with *M.luteus*. Transcript abundance of in S2 Schneider cells was compared to the non-challenged sample. The 24 hours challenge was replicated tree times.

DISCUSSION

1. DNase IIIβ classification

In this study, we have characterized a novel nuclease from *Drosophila melanogaster*. Based on a phlyogenetic analysis, DNase IIIβ, was classified as member of the family of the duplex specific nucleases (DS_NUCs). Initially, when a sequence analysis of this protein was performed, we found that this enzyme contains a stretch of 22 amino acid sequences that is called the beta-beta-alpha metal finger, which structurally is made up of two beta-strands and an alpha helix. This correlated with our initial thoughts that DNase IIIβ belongs to the Serratia family of nucleases (SFN). *Serratia marcescens* endonuclease is a well-studied example of this family for which structural information is available (Miller et al., 1999; Shlyapnikov et al., 2000). However, after further study of the SFN family done by Amisinova *et al* the DNase IIIβ sequence was classified as part of the DS_NUCs family.

2. DNase IIIβ shares some structural and enzymatic similarities with *S. marcescens* endonuclease

Alignment of the DNase IIIβ putative active center (Figure 9) with other DNases of the DNA/RNA non-specific nucleases shows the presence of the conserved Arginine, Glycine, Histidine (RGH) triad found in most DNases characterized as well as all of the conserved residues are present in the DNase IIIβ. The active site of *S. marcescens* endonuclease involves several highly conserved amino acid residues: His143, Asn174, Glu182, plus the Asn119 residue ligand of the magnesium atom (Miller, Schäfer, Ghosh). These four amino acids residues are highly conserved among DNases and equidistantly localized in DNase IIIβ, forming the catalytic site for phosphodiester bond cleavage.

In addition, the hypothetical 3D structure of novel DNase IIIb was determined by comparison with non-specific endonuclease from *Serratia marcescens*. Monomer of endonuclease from *Serratia marcescens* was selected as comparison structure, based on software criteria. The software compares amino acid sequence based on models of homologues proteins of known structure (Figure 10)**.** This fact suggests that DNase IIIβ shares similar structure with *S. marcescens***.** Structurally, both nucleases show quite similar active site geometry, suggesting that both enzymes might have similar mechanisms of action on DNA substrates.

3. Expression of GST-DNase IIIβ-$(His)_6$ protein construct

Full-length tagged recombinant DNase IIIβ was amplified by PCR and sub-cloned in pGEX-K vector for expression as a fusion protein with a $(His)_6$ tag at the C-terminal and a GST tag at the N-terminal. Recently, a full-length tagged recombinant DNase IIIβ was produced after IPTG induction in BL21 (DE3) pLysS bacteria. Our studies showed that a reduction in the induction temperature from 37 °C to 16 °C resulted in a decrease in the yield of the protein.

4. Purification of GST-DNase IIIβ-$(His)_9$

In this part of the study, the main goal was to design a dual affinity tag purification system to enable purification of the tagged recombinant in a non-denatured and non-aggregated soluble state for biochemical studies. Therefore, we have substituted the standard $(His)_6$ tag with a $(His)_9$ tag with the aim of increasing binding of the full-length tagged recombinant DNase IIIβ. The GST tag was chosen because of its efficient translation initiation in *E.coli* systems (Waugh, 2005). It may also help to enhance the solubility of the tagged recombinant DNase IIIβ protein during its lysis and purification.

Recombinat DNase IIIβ expressed in *E.coli* was localized in both the inclusion bodies and in the soluble fraction. However, DNase IIIβ could be purified in its native state as GST-$(His)_6$-tagged recombinant protein using a GST affinity purification. Although our attempts to express functional recombinant protein presented several challenges, we were able to purify DNase IIIβ. The identity of this recombinant protein was confirmed by immunobloting using anti-GST antibodies. Lastly, after the final step of purification, DNase IIIβ was found to have nuclease activity at pH 5-7 in the presence of Mg^{2+} after different time intervals incubation periods (1, 3, and 8 hours) at 37 °C.

5. Quantitative real-time PCR analysis

Quantitative real-time PCR was included in this study in order to assess the relative abundance of CG9989 transcripts in Schneider 2 (S2) cells in normal conditions and when challenged with the Gram positive bacteria *Micrococcus luteus*. The study was normalized using the housekeeping gene Ribosomal Protein 15a (RP15a). As a positive control for bacterial challenge, we measured changes in Drosomycin transcripts since this antimicrobial peptide is reported to be expressed in S2 cells by Drosophila Peptide Atlas (http://www.mop.uzh.ch/drosophila-peptideatlas/cgi/PeptideAtlas/GetPeptides?_tab=1). In addition, it has been shown that adult wild-type flies show a 10 fold increase in Drosomycin transcripts 24 hours after infection with *M. luteus* in comparison with non-infected adult flies (Lemaitre, B., *et al*., 1997).

Our results show that the relative abundance of CG9989 transcripts did not significantly increase in S2 cells at 24 hours post-challenge compared to non-infected control. Interestingly, previous real time PCR analyses performed in our lab with adult flies revealed an over-

expression of the CG9989 gene at 24 and 48 hours post-infection with *M. luteus*. However, the same bacterial challenge performed on S2 cells at 24 hours did not reveal any induction. The Drosomycin transcripts increased a 3.15 fold in comparison with the non-infected S2 cells, indicating that *M. luteus* does induce the production of antimicrobial peptides. However, Drosomycin is a gene that should be up regulated by 10 fold as previously reported (Lemaitre, B., *et al*., 1997) and it only showed a 3.15 fold increase in S2 cells. The low expression of Drosomycin and CG9989 in S2 cells after bacterial challenge could be attributed to the fact that these cells function as pre-phagocytes while the fat body is the organ in charge of the production of antimicrobial peptides in the fly. Therefore, we would expect to have a higher expression of both genes in the whole fly compared to S2 cells. An alternative explanation for the low-expression of Drosomycin and CG9989 is the use of paraformaldehyde to fix *M. luteus*. It is possible that fixed bacteria do not stimulate antimicrobial peptide gene induction as robustly as intact bacteria. Finally, recent studies performed in our lab in S2 cells demonstrates a mutation in the *relish* binging site inside the promoter of the CG9989 gene. This could explain the low-expression of CG998 gene in S2 cells since the Relish transcription factor is involved in antimicrobial peptide gene induction (Matova, N. and Anderson, K.V., 2009)

REFERENCES

Anisimova V.E. *et. al*, (2008). Isolation, characterization and molecular cloning of Duplex-Specific Nuclease from the hepatopancreas of the *Kamchatka* crab. *BMC* Biochemistry, 9:14.

Baker K.P. *et. al*, (1998). Molecular cloning and characterization of human and murine DNase II. Gene 215 (1998) 281–289.

Belfort M., *et al*, (2005). Homing Endonucleases and Inteins. Springer. First edition. pp. 2-4.

Benedik, M.J., and Strych, U. (1998) *Serratia marcescens* and its extracellular nuclease. FEMS Microbiology Letters 165. pp. 1-13.

Bernardi, G., 1971. Spleen acid deoxyribonuclease. In: Boyer, P.D. (Ed.), The Enzymes. Academic Press, New York, Vol. 4, pp. 271–287.

Biertumpfel, C., Yang,W. and Suck,D. (2007) Crystal structure of T4 endonuclease VII resolving a Holliday junction. Nature, 449, 616–620.

Calvo E. and Ribeiro J.M.C. (2006). A novel secreted endonuclease from *Culex quinquefasciatus* salivary glands. The journal of Experimental Biology 209, pp 2651-2659.

Chang, M.C., Chang, S.Y., Chen, S.L. and Chuang, S.M. (1992) Cloning and expression in Escherichia coli of the gene encoding an extracellular deoxyribonuclease (DNase) from Aeromonas hydrophila. Gene, 122, 175-180.

De Gregorio E, Spellman P.T, Rubin G.M , and Lemaitre B. (2001). Genome-wide analysis of the Drosophila immune response by using oligonucleotide microarrays. 12590–12595; PNAS; vol. 98; no. 22.

Dhananjaya, B.L., Souza C.J., (2010) An overview on nucleases (DNase, RNase, and phosphodiesterase) in snake venoms. Biochemistry (Mosc). 2010 Jan;75(1):1-6.

Dodd, H.N. and Pemberton, J.M. (1996) Cloning, sequencing and characterization of the nucH gene encoding an extracellular nuclease from Aeromonas hydrophila JMP636. J. Bacteriol., 178, 3926-3933.

Evans CJ, Merriam JR, Aguilera RJ. (2002). Drosophila acid DNase is a homolog of mammalian DNase II. Gene 295. 61–70.

Evans C.J and Aguilera R.J. (2003). DNase II: genes, enzymes and function. Gene 322. 1-15.

Flick,K.E., Jurica,M.S., Monnat,R.J. Jr and Stoddard,B.L. (1998) DNA binding and cleavage by the nuclear intron-encoded homing endonuclease I-PpoI. Nature, 394, 96–101.

Focareta,T. and Manning,P.A. (1991) Distinguishing between the extracellular DNases of Vibrio cholerae and development of a transformation system. Mol. Microbiol., 5, 2547-2555.

Galburt,E.A., Chevalier,B., Tang,W., Jurica,M.S., Flick,K.E., Monnat,R.J., Jr, and Stoddard,B.L. (1999) A novel endonuclease mechanism directly visualized for I-PpoI. Nat. Struct. Biol., 6, 1096–1099.

Garcia A.D., Aravind, L., Koonin, E.V., and Moss, B. (2000). Bacterial-type DNA Holliday junction resolvases in eukaryotic viruses. PNAS, 107, Vol.97, no. 16, pp.8926-8931.

Ghosh, M., Meiss, G., Pingoud, A., London, R.E., and Pedersen, L.C. (2005). Structural Insights into the Mechanism of Nuclease A, a Metal Nuclease from Anabaena. The Journal of Biological Chemistry. Vol. 280, No. 30, Issue of July 29, pp. 27990–27997

Hedgecock EM, Sulston JE, Thomson JN. Mutations affecting programmed cell deaths in the nematode *Caenorhabditis elegans*. Science 1983;220:1277-1279. [PubMed: 6857247].

Holliday, R. (1964). A mechanism for gene conversion in fungi. Genet. Res. 5, pp. 282–304.

Jekel, M., and Wackernagel, W. (1995) The periplasmic endonuclease I of Escherichia coli has amino-acid sequence homology to the extracellular DNases of Vibrio cholerae and Aeromonas hydrophila. Gene, 154, 55-59.

Lemaitre, B., Reichhart, J.M., and Hoffmann, J.A. (1997). *Drosophila* host defense: Differential induction of antimicrobial peptide genes after infection by various classes of microorganisms. Immunology. Vol.94, pp. 14614-14614.

Li,C.L., Hor,L.I., Chang,Z.F., Tsai,L.C., Yang,W.Z. and Yuan,H.S. (2003) DNA binding and cleavage by the periplasmic nuclease Vvn: a novel structure with a known active site. EMBO J., 22, 4014–4025.

Lyon C.J. *et. al*, (2000). The *C. elegans* apoptotic nuclease NUC-1 is related in sequence and activity to mammalian DNase II. Gene 252. 147–154.

Matova, N. and Anderson, K.V. (2009) Drosophila Rel proteins are central regulators of a robust multi-organ immune network. Journal of cell science. Vol 123, pp. 627-633.

Meiss, G. Franke, I., Gimadutdinow, O., Urbanke, C., and Pingoud, A. (1998). Biovhemical Characterization of Anabaena sp. strain PCC 7120 non-specific nuclease NucA and its inhibitor NuiA. Eur.J. Biochem. 251, 924-934.

Moulard, M., Condemine, G., and Robert-Baudouy,J. (1993). Characterization of the nucM gene coding for a nuclease of the phytopathogenic bacteria Erwinia chrysanthemi. Mol. Microbiol., 8, 685-695.

Pommer,A.J., Wallis,R., Moore,G.R., James,R. and Kleanthous,C. (1998) Enzymological characterization of the nuclease domain from the bacterial toxin colicin E9 from Escherichia coli. Biochem. J., 334 (Pt 2), 387–392.

Shagin, D.A., et al. (2002). A novel method for SNP detection using a new duplex specific nuclease from crab hepatopancreas. Genome Res. 12, pp 1935-1942.

Shen,B.W., Landthaler,M., Shub,D.A. and Stoddard,B.L. (2004) DNA binding and cleavage by the HNH homing endonuclease I-HmuI. J. Mol. Biol., 342, 43–56.

Sokolowska M *et al*, (2009). Crystal structure of the ββα-Me type II restriction endonuclease Hpy99I with target DNA. Nucleic Acids Research, 2009, Vol. 37, No. 11, pp. 3799–3810.

Sulston JE. (1976).Post-embryonic development in the ventral cord of *Caenorhabditis elegans*. Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences, Vol. 275, No. 938, pp. 287-297.

CURRICULUM VITAE

Brenda Cristina Anchondo was born in Chihuahua, Mexico. The fisrt daughter of Wenceslao Anchondo and Socorro Munoz, she graduated from the Universidad Autonoma de Chihuahua, in Chihuahua, Mexico, in the fall of 2005. While pursuing a bachelor's degree in Chemistry in Bacteriology/Parasitology, she worked under the direction of Dr. Quintin Rascon developing a thesis titled: "Clonacion y Elaboracion de Mapas de Restriccion de Segmentos del Genoma de Cloroplasto de Maiz y Pasto"-Cloning, sequencing, and development restriction maps of corn and grass chloroplast genomes- this research resulted in the generation of novel transgenic plants that express nutrient rich peptides. After she graduated, she worked in the "Centro Medico de Especialidades"-hospital as a Medical and Clinical Laboratory Scientist, while working there she conducted chemical analysis of body fluids, including blood, urine, and spinal fluids, to determine the presence of normal and abnormal components of about 80 patients in a daily basis. In the fall of 2007, she entered the Graduate School at The University of Texas at El Paso to pursue a Master's degree. While pursuing the degree she worked as a Teaching Assistant for the General Microbiology Laboratory, where she prepared and delivered laboratory lectures to undergraduate students on topics such as Microbiology. In addition, she also worked as a Research Assistant under the direction of Dr. Renato Aguilera where she studied *Drosophila* and *dnase* II-RNAi *Drosophila* immune system genes and found a gene that encodes an important immune peptide which results in the characterization of a nuclease using basic molecular biology techniques such as: Cloning, gel electrophoresis, plasmid isolation, Polymerase Chain Reaction (PCR), SDS-PAGEs, Western Blotting, and chromatography-protein purification. She also participated in conference proceedings during Graduate Scholl, she

assisted to the SACNAS Research Expo 2009, in Dallas, TX, where she presented a poster titiled: Cloning and expression of a novel infection/stress activated fly endonuclease.

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