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RESEARCH ARTICLE

Dual silencing of long and short *Amblyomma americanum* acidic chitinase forms weakens the tick cement cone stability

Tae K. Kim, Janet Curran and Albert Mulenga*

ABSTRACT

This study demonstrates that *Amblyomma americanum* (*Aam*) constitutively and ubiquitously expresses the long (L) and short (S) putative acidic chitinases (Ach) that are distinguished by a 210 base pair (bp) deletion in *Aam*Ach-S. Full-length *Aam*Ach-L and *Aam*Ach-S cDNA are 1959 and 1718 bp long, containing 1332 and 1104 bp open reading frames that code for 443 and 367 amino acid residues proteins with the former predicted to be extracellular and the latter intracellular. Both *Aam*Ach-L and *Aam*Ach-S mRNA are expressed in multiple organs as revealed by qualitative RT-PCR analysis. Furthermore, quantitative reverse transcription polymerase chain reaction analysis revealed that *Aam*Ach-L mRNA was downregulated in the mid-gut, but was unchanged in the salivary gland and in other organs in response to feeding. Of significant interest, *Aam*Ach-L and/or *Aam*Ach-S functions are probably associated with formation and/or maintenance of stability of *A. americanum* tick cement cone. Dual RNA interference silencing of *Aam*Ach-L and/or *Aam*Ach-S mRNA caused ticks to loosely attach onto host skin as suggested by bleeding around tick mouthparts and ticks detaching off host skin with a light touch. *Aam*Ach-L may apparently encode an inactive chitinase as indicated by *Pichia pastoris*-expressed recombinant *Aam*Ach-L failing to hydrolyse chitinase substrates. Unpublished related work in our laboratory, and published work by others that found *Aam*Ach-L in tick saliva, suggest that native *Aam*Ach-L is a non-specific immunoglobulin binding tick saliva protein in that r*Aam*Ach-L non-specifically bound rabbit, bovine and chicken non-immune sera. We discuss findings in this study with reference to advancing knowledge on tick feeding physiology.

KEY WORDS: *Amblyomma americanum*, Putative acidic chitinase, Glycoside hydrolase 18 family, Tick cement, Tick feeding physiology

INTRODUCTION

Ticks are important ectoparasites that transmit diverse animal and human disease agents such as protozoa, bacteria, spirochaetes and viruses (Jongejan and Uilenberg, 1994) and are considered second to mosquitoes in terms of the impact on medical and veterinary transmitted diseases (Sonenshine, 1993; Jongejan and Uilenberg, 2004). *Amblyomma americanum* (Linnaeus) is an important tick distributed in North America. This tick transmits multiple tick-borne disease (TBD) agents, *Ehrlichia chaffensis* (Anderson et al., 1993), *E. ewingii* (Wolf et al., 2000), *Francisella tularensis* (Taylor et al., 1991), an unknown causative agent of the disease called southern tick associated rash illness (STARI) (James et al., 2001), *Rickettsia amblyommii* (Apperson et al., 2008) and *Theileria cervi* (Laird et al., 1988). *Amblyomma americanum* has also been linked to transmission of the recently described human tick-borne Heartland virus (Savage et al., 2013). The distribution of *A. americanum* has expanded from southeastern United States (Mixson et. al., 2006) to northeastern regions as far as Maine (Keirans and Lacombe, 1998). The expanding geography and its role as a vector of numerous human TBD agents makes this tick important in public health policy. Currently methods of tick control and prevention of human and animal TBD infections is dependent on use of chemical acaricides. While acaricides are effective in the short term, they do not provide a permanent solution due to multiple limitations such as ticks developing resistance to acaricides, environmental contamination, cost of developing new acaricides, and the inconvenience of application procedures. These limitations have necessitated the search for alternative novel tick control methods that will provide a permanent solution (Graf et al., 2004; de la Fuente and Kocan, 2006; de la Fuente et al., 2007). Immunization of animals against tick infestation has been validated as a sustainable alternative tick control method (Opdebeeck et al., 1988; Willadsen, 2004; de la Fuente et al., 2010). The pre-requisite to this is a deeper understanding of tick feeding biology and physiology as a means to discover weak links in tick biology that can be targeted for tick vaccine development. In our laboratory we are studying molecular events of early stage tick feeding physiology that precedes key facets of tick parasitism, TBD agent transmission, blood meal uptake, and reproduction. Towards this goal subtractive hybridization analysis was used to identify 40 *A. americanum* (*Aam*) genes that were differentially up-regulated in ticks that were preparing to start feeding (Mulenga et al., 2007). One of these 40 genes is the focus of this study, and is a putative *A. americanum* tick acidic chitinase (*Aam*Ach).

Chitinases are enzymes that hydrolyse the β-1,4 glycosidic linkages of *N*-acetylglucosamines primarily found in chitin. Chitin is an insoluble structural polysaccharide that is important as a supporting element in arthropod exoskeleton (Neville et al., 1976), fungal and bacterial cell walls (Debono and Gordee, 1994; Gomaa, 2012), microfilaria sheath (Araujo et al., 1993) and the lining of the digestive tracts of many arthropods (Souza-Neto et al., 2003; Zimoch et al., 2005; Khajuria et al., 2010). Arthropod development and morphogenesis rely on remodeling chitin and in the process requires chitin synthases and chitinases to control this process (Merzendorfer and Zimoch, 2003). Insect chitinases are also involved in cuticle turnover, digestion and degradation of peritrophic membrane during molting (Kramer and Muthukrishnan, 1997; Merzendorfer and Zimoch, 2003; Rao et al., 2004). In fungi, chitinases were involved in autolysis, nutrition, morphogenesis and parasitism (Ghormade et al., 2000). Most bacterial chitinases are involved in degrading chitin to provide nitrogen and carbon (Patil et al., 2000). In plants, chitinases were involved in defence (Gooday,

Texas A&M University AgriLife Research, Department of Entomology, 2475 TAMU, College Station, TX 77843, USA.

*Author for correspondence (a-mulenga@tamu.edu)

Received 13 May 2014; Accepted 21 July 2014

The Journal of Experimental Biology (2014) doi:10.1242/jeb.107979

1999) and morphogenesis (Grover, 2012), while in nematodes, chitinases were associated with development (Geng et al., 2002; Tachu et al., 2008). Mammals may not synthesize chitin, but the human genome encodes eight chitinases that are involved in T-cell-mediated inflammation and asthma (Kawada et al., 2007; Reese et al., 2007) and tissue remodeling and injury (Lee et al., 2011). Emerging data suggest that chitinases are important to tick physiology. In the tick *Haemaphysalis longicornis*, immunization of rabbits with recombinant virus expressing a chitinase was used as a bioacaricide, which caused reduced feeding efficiency (You et al., 2003; Assenga et al., 2006), and prevented molting (You and Fujisaki, 2009). The purpose of this study was to gain insight into the role(s) of a tick feeding stimuli responsive putative acidic chitinase (Ach) in *A. americanum* (*Aam*) tick feeding physiology. We demonstrate that *A. americanum* expresses the long and short *Aam*Ach and that both forms may be important to maintenance of stability of *A. americanum* tick cement cone.

RESULTS

Amblyomma americanum expresses long and short putative *Aam*Ach forms

A partial cDNA encoding putative acidic chitinase was identified among *A. americanum* feeding stimuli responsive genes (Mulenga et al., 2007). In this study rapid amplification of cDNA ends (RACE) was used to amplify the full-length cDNA (not shown). While analysing DNA sequences of cDNA clones, we identified long (L) 1959 base pair (bp) (GenBank accession no. KF819831) and short (S) 1718 bp (GenBank accession no. KF819830) forms, with *Aam*Ach-S characterized by a 210 bp deletion (Fig. 1A). *Aam*Ach-L and *Aam*Ach-S full-length cDNA respectively contain 1332 and 1104 bp open reading frame (ORF) that encodes 443 and 367 amino acid residue proteins (Fig. 1B). To verify if the 210 bp deletion was physiological and not a sequencing error, specific *Aam*Ach-L and *Aam*Ach-S polymerase chain reaction (PCR) primers were designed around the deleted region, as indicated in

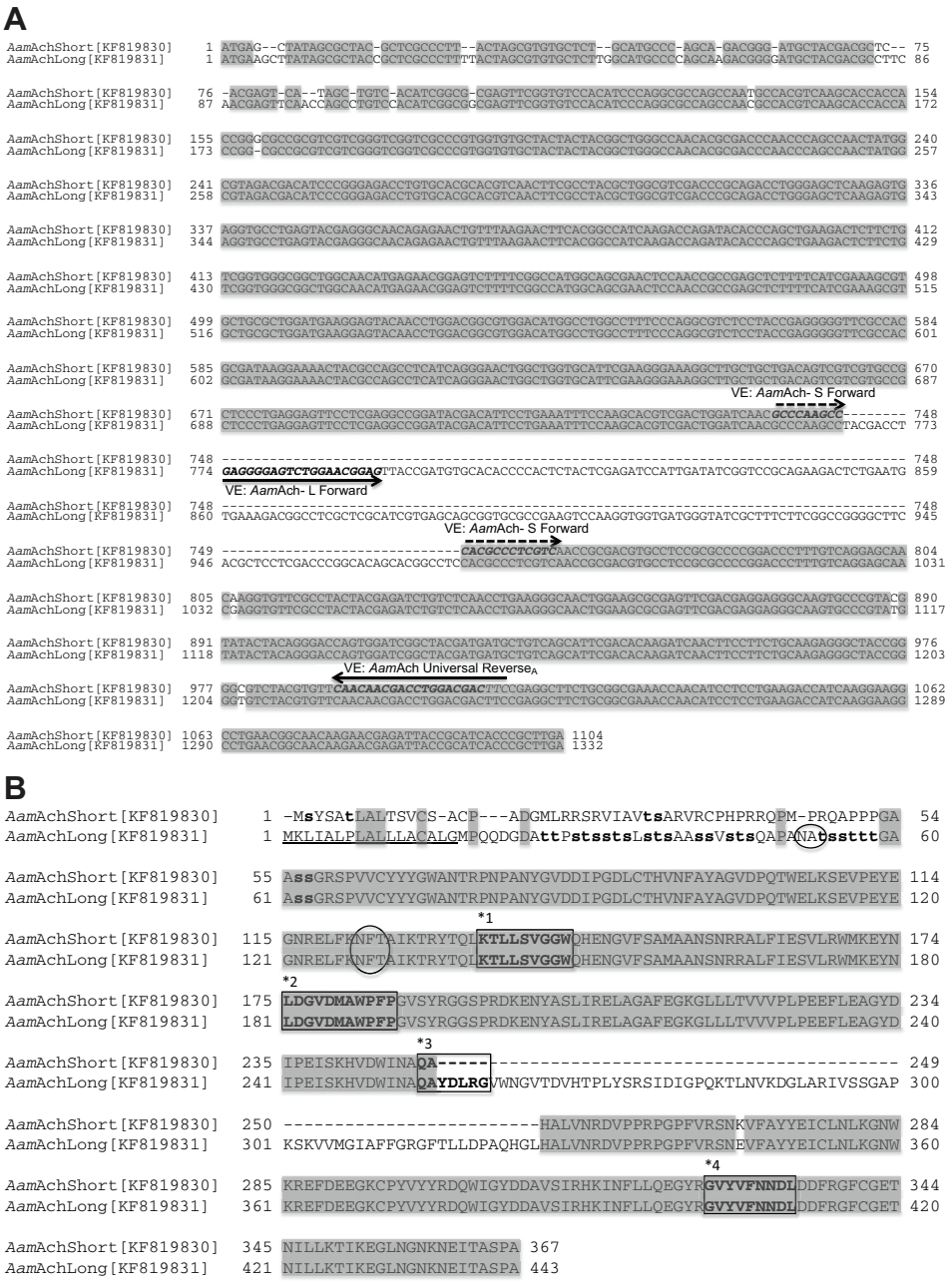

Fig. 1. ClustalW pairwise alignment of *A. americanum* long (L) and short (S) nucleic acid and amino acid sequence open reading frame. Sequences were aligned using the T-coffee sequence alignment tool in MacVector analysis software. (A) The interrupted line denotes the 210 base pair deletion. VE denotes PCR primers to qualitatively validate *Aam*Ach-L and *Aam*Ach-S expression (see Fig. 2). Priming sites are in italic bold type. Specific forward primers are noted and marked by interrupted (*Aam*Ach-S) and continuous (*Aam*Ach-L) line arrows. (B) The four glycoside hydrolase (GH)-18 family consensus amino acid motifs are shown within boxes and marked with numbered asterisks: *1 denotes the motif K(F/V)M(V/L/I)AVGGW, *2 denotes the motif FDG(L/F)DLDWE(Y/F)P, *3 denotes the motif M(S/T)YDL(R/H)G, and *4 denotes the motif GAM(T/V)WA(I/L)D. Predicted *O*-linked (bold lower case) and *N*-linked (circled) glycosylation sites in *Aam*Ach-L and *Aam*Ach-S are marked. A 17 amino acid signal peptide for *Aam*Ach-L is underlined.

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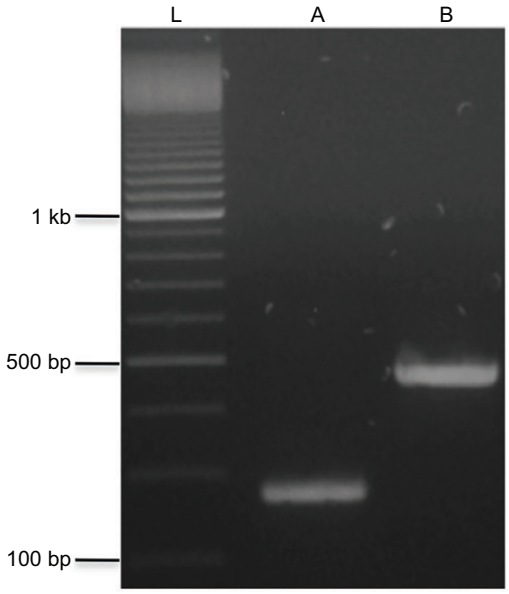

Fig. 2. PCR validation of *A. americanum* long and short putative acidic chitinase transcripts. PCR amplification of *Aam*Ach-L and *Aam*Ach-S transcripts was done using the specific primers marked in Fig. 1A. PCR products were resolved on a 2% agarose gel containing 1 μg ml^{-1} ethidium bromide. Lanes A and B show ~300 and 500 bp bands for *Aam*Ach-S and *Aam*Ach-L respectively. Lane L shows the 100 bp ladder.

Fig. 1A. Forward primers were designed to specifically anneal *Aam*Ach-L or *Aam*Ach-S with the reverse primers annealing to both forms. Consistent with the observed 210 bp deletion (Fig. 1A), PCR primers amplified ~500 and 300 bp *Aam*Ach-L and *Aam*Ach-S cDNA fragments, respectively (Fig. 2), confirming that both forms were expressed in *A. americanum*.

Both *Aam*Ach-L and *Aam*Ach-S isoforms belong to the glycoside hydrolase-18 (GH-18) family

When subjected to amino acid motif scanning on the ScanProsite Expasy server (de Castro et al., 2006) *Aam*Ach-L and *Aam*Ach-S did not retrieve any matches (not shown). However, when scanned against entries in GenBank, both forms showed identity to annotated chitinase and chitinase-like proteins in the glycoside hydrolase-18 (GH-18) family with amino acid identity levels ranging from 40 to 85% (not shown). When scanned on the SignalP server version 4.01 (Petersen et al., 2011), a 17 amino acid residue putative signal peptide was predicted in *Aam*Ach-L, but not *Aam*Ach-S (Fig. 1B). The GH-18 family is characterized by: (1) signal peptide, (2) Ser/Thr-rich linker regions that may be heavily glycosylated located in the N- or C-terminal region linking the signal peptide, catalytic domain and chitin binding domain (Arakane et al., 2003; Huang et al., 2012), (3) one to several GH-18 catalytic domains that have conserved DXDXE and/or SXGG motif (Henrissat, 1991; Watanabe et al., 1993; Tsuji et al., 2010), and (4) none to five chitin binding domains characterized by the CX_{67}-FX_{67} -CX_2 -YX_7 -CX_{919}-CX_{46}-FX_{47}-CX_{512}-C motif (Fuhrman et al., 1995; Tellam, 1996). In arthropods, the typical GH-18 chitinase catalytic domain is characterized by four signature amino acid sequence motifs including active site 'FDG(L/F)DLDWE(Y/F)P' containing the conserved DXDXE motif (Thomas et al., 2000; Lu et al., 2002), 'K(F/V)M(V/L/I)AVGGW' containing the conserved SXGG motif, 'M(S/T)YDL(R/H)G' and 'GAM(T/V)WA(I/L)D' (Arakane and Muthukrishnan, 2010). Except for putative chitin binding domains, the four consensus amino acid motifs and putative glycosylation sites are present in both *Aam*Ach-L and *Aam*Ach-S (Fig. 1B). Of the four amino acid motifs, three ('LDGVDMAWPFP', 'QAYDLRG' and 'GVYVRNND') are conserved up to 55, 67 and 38%, respectively, while the fourth motif 'KTLLSVGFW' is 71% conserved in *Aam*Ach-L but absent in *Aam*Ach-S. As shown in Fig. 1B, both *Aam*Ach-L and *Aam*Ach-S have 2 and 1 putative *N*-linked (circled) and 24 and 6 *O*-linked glycosylation sites (marked as bold lowercase letters) located in the N-terminal region, which may indicate a S/T linker region connecting the signal peptide with the catalytic domain.

Phylogeny relationship of *Aam*Ach-L and *Aam*Ach-S amino acid sequences with other tick sequences

Fig. 3 summarizes the phylogeny relationship of *Aam*Ach-L and *Aam*Ach-S putative proteins with other tick GH18-like chitinases

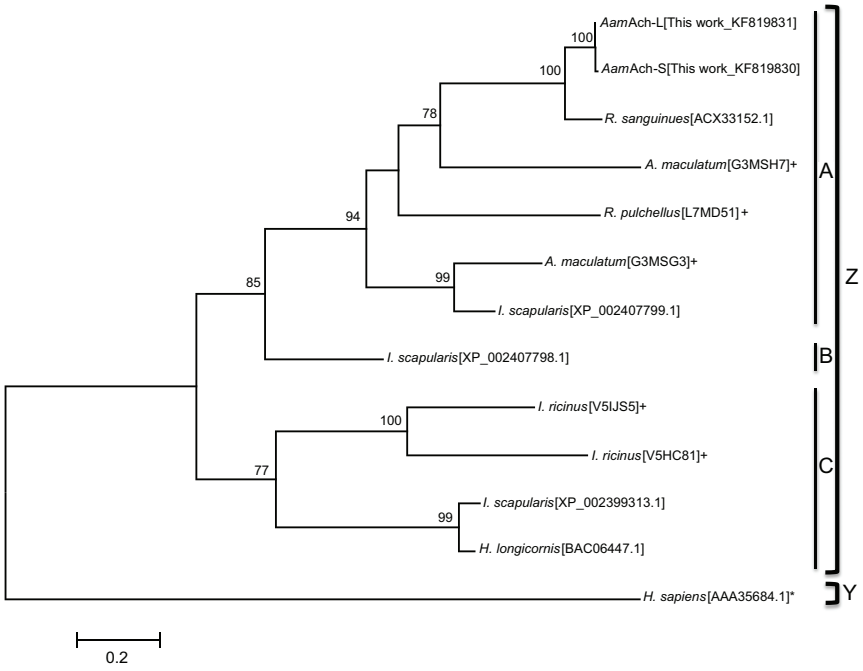

Fig. 3. Phylogeny comparison of long and short putative *A. americanum* acidic chitinase amino acid sequences with other tick GH-18 chitinase sequences. A guide phylogeny tree of *Aam*Ach-L and *Aam*Ach-S putative protein sequences and GH-18 chitinase sequences of other tick species was constructed using the maximum likelihood method. Species shown: *Amblyomma maculatum*, *Rhipicephalus sanguineus*, *Rhipicephalus pulchellus*, *Ixodes scapularis*, *Ixodes ricinus*, *Haemaphysalis longicornis* and *Homo sapiens*. The number at each node represents bootstrap values that signify the level of confidence in the branch. A denotes cluster A, B denotes cluster B, C denotes cluster C, Z denotes the GH-18 like chitinase group and Y denotes the outgroup of human chitobiase, *H. sapiens* [AAA35684.1]*. Sequences were retrieved from NCBI, with the exception of UniProt sequences denoted with +. Scale bar represents estimated phylogenetic distance in substitutions per site.

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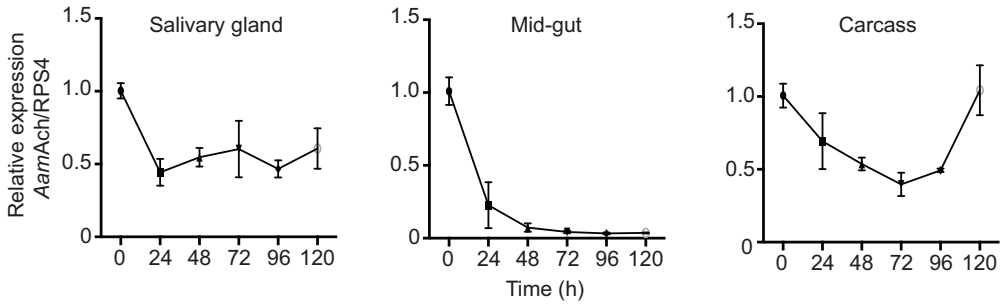

Fig. 4. Temporal and spatial expression analysis of *Aam*Ach-L mRNA during 120 h post-attachment. *Aam*Ach-L mRNA expression was subjected to quantitative RT-PCR expression analysis in unfed, 24, 48, 72, 96 and 120 h fed tick dissected tissues: salivary gland, mid-gut and carcass using qRT-PCR primers (forward: 5′-CTGGAACGGAGTTACCGATGTGC-3′ and reverse: 5′-TGCGGACCGATATCAATGGAT-3′). The average relative expression levels were plotted along with the standard error of the mean for each tissue. To determine the *Aam*Ach-L mRNA relative expression (*y*-axis), data were analysed using the $2^{-\Delta\Delta C_T}$ method comparing with RPS4 from three biological replicates of different tissues in relation to time of feeding (*x*-axis).

downloaded from GenBank and Uniprot (marked with [+]). The tree out-rooted from *Homo sapiens* GH-18 chitobiase (accession number: AAA35684.1) was constructed by the maximum likelihood method set to default parameters in the Molecular Evolutionary Genetics Analysis (MEGA) 5.2.2 online software (http://www.megasoftware.net) (Fig. 3). As shown in Fig. 3, sequences segregated into three clusters: A, B, and C supported by 94, 85 and 77% bootstrap values, respectively. Both *Aam*Ach-L and *Aam*Ach-S amino acid sequences segregated with *Rhipicephalus sanguineus* (ACX33152.1), *Amblyomma maculatum* (G3MSH7, G3MSG3), *Ixodes scapularis* (XP_002407798.1, XP_002407799.1) and *R. pulchellus* (L7MD51). Within cluster A, *Aam*Ach-L and *Aam*Ach-S show a respective 86 and 83% amino acid identity to *R. sanguineus* (ACX33152.1). However, when compared with remaining sequences, amino acid identity levels decreased to between 34 and 51% in cluster A and 24–45% in clusters B and C (not shown).

Both *Aam*Ach-L and *Aam*Ach-S mRNA are ubiquitously expressed

Qualitative reverse transcription PCR (qRT-PCR) using PCR primers in Fig. 1A revealed that both *Aam*Ach-L and *Aam*Ach-S mRNA were expressed through the first 5 days of feeding in all tested tick organs (not shown). Attempts to find optimal *Aam*Ach-S qRT-PCR primers for *Aam*Ach-S were not successful. Given the importance of the salivary gland and mid-gut to tick physiology, we determined quantitative *Aam*Ach-L mRNA expression levels in these organs (Fig. 4). For qRT-PCR analysis, we synthesized cDNA of dissected salivary glands (SG), mid-gut (MG) and carcass (CA) (tick remnant after removal of SG and MG) pooled from ten unfed and 24 h fed, eight 48–96 h fed, and five 120 h fed ticks in triplicate. As summarized in Fig. 4, *Aam*Ach-L displayed a dichotomous expression pattern. In MG, *Aam*Ach-L mRNA is significantly down-regulated in response to tick feeding activity dropping to near zero by the 120 h time point. In contrast, in the SG and CA (Fig. 4), *Aam*Ach-L mRNA expression levels did not significantly change through the 120 h feeding time point when compared with unfed ticks. Please note that exceptions to this were between unfed versus 24 h (P=0.0377) or 96 h (P=0.0477) in SG, and unfed versus 72 h (P=0.0277) in CA time points where transcript abundance was significantly higher in unfed ticks.

Expression and affinity purification of recombinant *Aam*Ach-L in *Pichia pastoris*

We are interested in characterizing extracellular proteins, and thus expression of recombinant (r) *Aam*Ach-S, predicted to function in intracellular protein, was not pursued. Fig. 5 summarizes expression and affinity purification of r*Aam*Ach-L in *Pichia pastoris*. Pilot expression showed a progressive daily increase in r*Aam*Ach-L expression levels, with the highest level observed at day 5 (Fig. 5A). Spent media of a 3 l large-scale expression was precipitated by ammonium sulphate saturation at 4°C and affinity purified using $NiCl_2^+$ charged Hi-Trap column (GE Healthcare Biosciences, Piscataway, NJ, USA). Following purification, elution fractions with

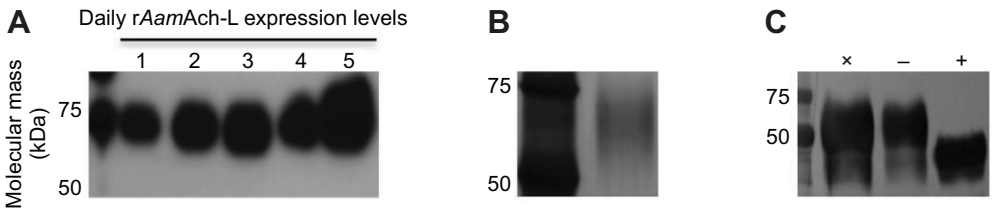

Fig. 5. Expression and affinity purification of recombinant *A. americanum* long acidic chitinase in *Pichia pastoris*. (A) Western blotting analysis of spent media using the antibody to C-terminus histidine tag to determine daily r*Aam*Ach-L expression levels during 5 days of culture (lanes 1−5). Construction of the expression plasmid, induction and validation of expression levels using antibody to the C-terminus hexa-histidine tag and affinity purification of putative r*Aam*Ach-L were performed as described in Materials and methods. (B) Validation of affinity purification of putative r*Aam*Ach-L using SDS-PAGE and silver staining. r*Aam*Ach-L was precipitated out of spent media by combination of ammonium sulphate saturation and affinity purified on $NiCl^{2+}$ charged columns under native conditions as described. (C) Western blotting analysis of r*Aam*Ach-L treated with a deglycosylation enzyme mix that removes both *O*- and *N*-linked glycans using antibodies to the C-terminus hexa-histidine tag. Lane X, r*Aam*Ach-L only; lane −, r*Aam*Ach-L treated with deglycosylation buffer components without deglycosylation enzyme mix; and lane +, r*Aam*Ach-L treated with deglycosylation buffer components and deglycosylation enzyme mix. Please note that the fraction used in Fig. 5C was relatively unpure when compared with the fraction in Fig. 5B, which was used in chitinase activity assays below.

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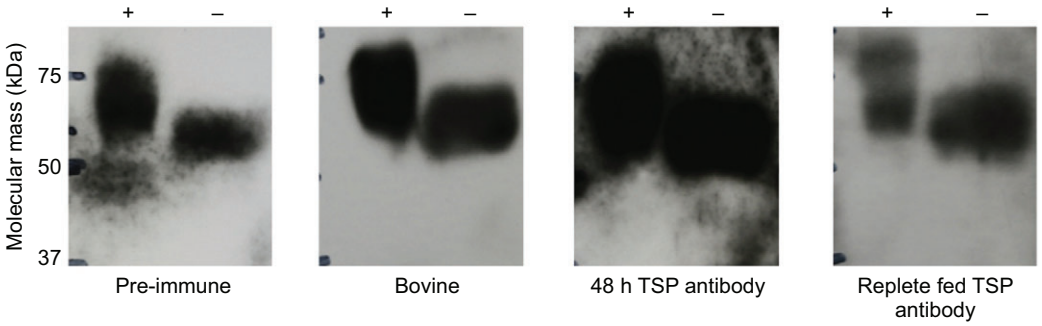

Fig. 6. Western blotting analysis to validate if native *Aam*Ach-L protein was injected into the host during tick feeding. Glycosylated (+) and deglycosylated (−) r*Aam*Ach-L western blots were exposed to non-immune sera of rabbit and bovine, and antibodies to 48 h and replete fed *A. americanum* tick saliva proteins (TSPs). Two thousandfold antibody dilutions were used for all blots.

relatively pure r*Aam*Ach-L as judged by SDS-PAGE with silver staining were pooled and concentrated using JumboSep™ centrifugal device filters (Pall Corporation, Port Washington, NY, USA). Concentrated proteins were subjected to SDS-PAGE on 10% acrylamide gel followed by silver staining to verify purity as shown in Fig. 5B. The predicted molecular mass of r*Aam*Ach is approximately ~53 kDa: comprised of ~2.5 kDa N-terminus fusion, ~47 kDa of mature r*Aam*Ach protein and ~3.5 kDa of C-terminal tag containing the hexa-histidine sequence. However, when r*Aam*Ach was electrophoresed on SDS-PAGE and developed by silver staining, a band at about 70 kDa is observed (Fig. 5B). Sequence analysis shown in Fig. 1B revealed that *Aam*Ach-L had two and 24 putative *N*- and *O*-linked glycosylation sites, respectively. Thus there is a possibility that the observed band shift from ~53 to ~70 kDa was due to post-translational glycosylation of r*Aam*Ach-L. When r*Aam*Ach-L was treated with a deglycosylation mix that cleaves off both *N*- and *O*-linked glycans, there was a downshift in the molecular weight to about ~60 kDa (Fig. 5C), confirming that r*Aam*Ach-L was glycosylated.

Native *Aam*Ach-L is a tick saliva protein that non-specifically binds immunoglobulins

Western blotting analysis of r*Aam*Ach-L using antibodies to 48 h (Chalaire et al., 2011) and replete fed (Mulenga et al., 2013) *A. americanum* tick saliva proteins was carried out to investigate the possibility of native *Aam*Ach-L being injected into the host during tick feeding. The expectation is that if native *Aam*Ach-L was immunogenic and injected into the host during tick feeding, r*Aam*Ach-L would specifically bind antibodies to 48 h and/or replete fed *A. americanum* tick saliva proteins. However, r*Aam*Ach-L non-specifically bound non-immune sera of rabbit (Fig. 6), bovine (Fig. 6) and chicken (not shown) as well as rabbit antibodies to 48 h and replete fed (Fig. 6) *A. americanum* tick saliva proteins. It is interesting to note that *Rhipicephalus microplus* saliva proteome contained a homolog to *Aam*Ach-L (Tirloni et al., 2014), while ongoing work in our laboratory has also found *Aam*Ach-L in *A. americanum* saliva proteome (T.K.K., L. Tirloni and A.M., unpublished observations), confirming that *Aam*Ach-L is a secreted tick saliva protein.

r*Aam*Ach-L has no chitinase activity

To determine if native *Aam*Ach-L was an active chitinase, r*Aam*Ach-L was subjected to chitinase function assay using a colorimetric substrate chitinase assay kit. There was no apparent chitinase activity against exochitinase activity substrates, 4-nitrophenyl *N*,*N*′-diacetyl-β-D-chitobioside and 4-nitrophenyl *N*-acetyl-β-D-glucosaminide or the endochitinase activity substrate, 4-nitrophenyl β-D-*N*,*N*′,*N*″-triacetylchitotriose. The positive control chitinase from *Trichoderma viride* provided in the assay kit showed activity against all substrates (not shown). The substrate hydrolysis assay buffer in the kit was at pH 4.8. To investigate the possibility that the kit's reaction buffer pH was not optimal for r*Aam*Ach-L activity, we assayed at a broad pH range of 4–6 as described by Carmody (Carmody, 1963). Under these conditions no activity was observed for r*Aam*Ach-L, while the positive control *T. viride* chitinase showed activity to all substrates (not shown).

Putative *Aam*Ach-S and *Aam*Ach-L are apparently important in maintenance of tick cement stability

To investigate the significance of *Aam*Ach-L and *Aam*Ach-S in tick feeding physiology, we synthesized double stranded RNA (dsRNA) targeting the region that was conserved between both forms as summarized in Fig. 7. Please note that the RNA interference (RNAi)

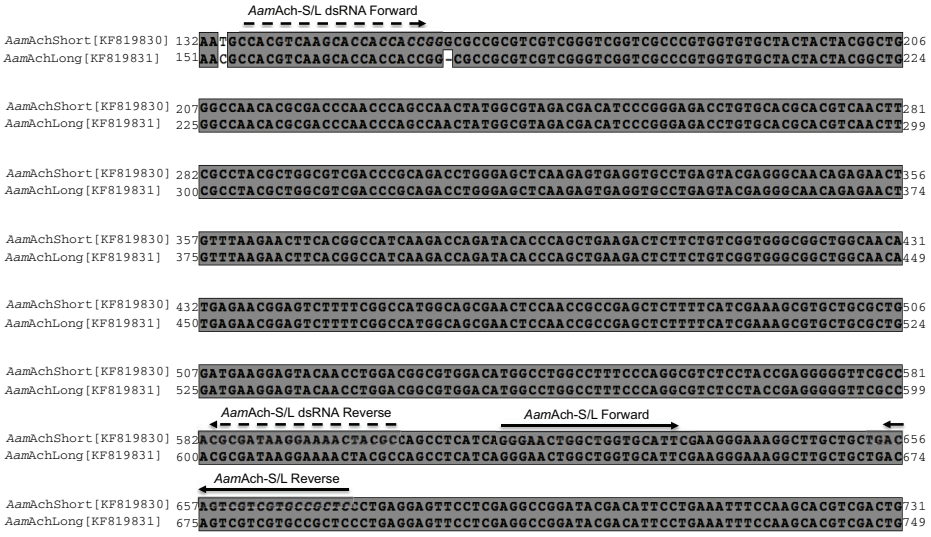

Fig. 7. Pairwise alignment of *Aam*Ach-L and *Aam*Ach-S cDNA regions used for *Aam*Ach-S/L dsRNA synthesis. Template cDNA was amplified by PCR using cloned *Aam*Ach-L plasmid as template. *Aam*Ach-S/L dsRNA synthesis priming sites labelled as *Aam*Ach-S/L dsRNA forward and reverse are noted and marked with interrupted arrows. Priming sites for validation of RNAi-mediated silencing of *Aam*Ach-S/L mRNA are labelled and marked with continuous line arrows.

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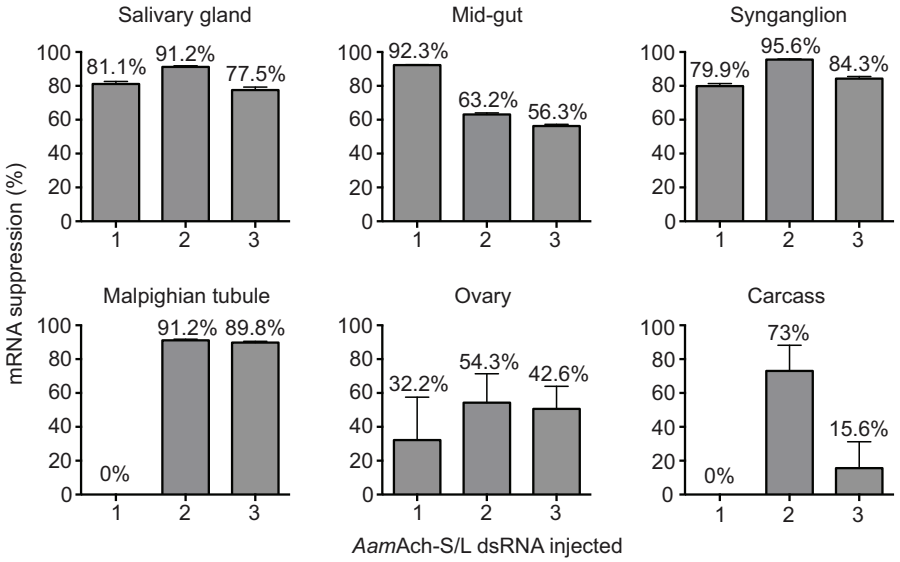

Fig. 8. Validating the disruption of *Aam*Ach-S/L mRNA in *Aam*Ach-S/L dsRNA injected ticks. Fifteen ticks were microinjected with 0.5–1 µl (2–3 µg µl^{-1}) of *Aam*Ach-S/L or GFP (control) dsRNA in nuclease free water. At 48 h post-attachment, three ticks per treatment of GFP-dsRNA injected control and *Aam*Ach-S/L-dsRNA injected ticks, were manually detached. Ticks were individually processed for mRNA extraction and then subjected to two-step quantitative RT-PCR using *Aam*Ach-S/L PCR primers on Fig. 7. Relative quantification (RQ) of *Aam*Ach-S/L mRNA expression was done using ABI 7300 software by the comparative C_T method ($\Delta\Delta C_T$). To determine the apparent level of *Aam*Ach-S/L mRNA suppression, the following formula was used: $S = 100 - (RQ^T/RQ^C \times 100)$, where S is mRNA suppression, and RQ^T and RQ^C are RQ of tissues of *Aam*Ach-S/L-dsRNA and GFP-dsRNA injected ticks, respectively.

silencing experiment was done twice. Results summarized in Fig. 8 represent qRT-PCR validation of *Aam*Ach-S/L mRNA suppression in three randomly sampled ticks that were injected with *Aam*Ach-S/L-dsRNA from the second experiment. *Aam*Ach-S/L mRNA was apparently suppressed by 77.5–91.2% in the salivary gland (SG), 79.9–96.6% in the synganglion (SYN), 56.3–92.3% in the mid-gut (MG), and 0–91.2% in Malpighian tubules (MT). Silencing in the ovary (OV) and carcass (CA) were 32.2–54.3 and 0–73%, respectively. It is interesting to note that while in tick 1 *Aam*Ach-S/L mRNA was suppressed in the SG, SYN, MG and OV, no silencing occurred in the MT and CA.

Investigation of tick feeding parameters revealed that silencing of *Aam*Ach-S/L mRNA did not affect the ability of ticks to attach onto host skin and start feeding because by visual inspection at 24 h after being placed on rabbits, 100% green fluorescent protein (GFP)-dsRNA and *Aam*Ach-S/L-dsRNA-injected ticks were attached (not shown). The tick RNAi-mediated silencing feeding phenotypes were documented every 24 h with pictures (Fig. 9). Fig. 9A,B presents documentation of physical phenotypes of *Aam*Ach-S/L-dsRNA and GFP-dsRNA injected ticks feeding on the rabbit host in two independent experiments. In both experiments leakage of blood around the mouthparts was observed by day 13 post-attachment in *Aam*Ach-S/L-dsRNA injected ticks (indicated by red arrows in Fig. 9A,B). No leakage of blood was observed in GFP-dsRNA injected control ticks. In the first experiment, bleeding was so intense that by the day 15, veterinarians at the Comparative Medicine Program (CMP) facility advised to halt the experiment due to the uncontrolled seepage of blood. Therefore, all the remaining ticks on the host were removed. In the second experiment bleeding had continued, but it was not as intense to stop the experiment when compared with the first experiment. While manually detaching *Aam*Ach-S/L-dsRNA injected ticks, they appeared to be weakly attached onto the host skin, in that ticks would fall off the host with a gentle touch.

Effects of *Aam*Ach-S/L silencing on feeding and fecundity are apparent but not statistically significant

As an index to measure the amount of blood imbibed by ticks, engorgement mass (EM) of replete fed and spontaneously detached *Aam*Ach-S/L-dsRNA and GFP-dsRNA injected ticks were determined. EM of *Aam*Ach-S/L-dsRNA and GFP-dsRNA injected ticks ranged from 362.7 to 751.2 mg (N=7) and 145.4 to 777.9 mg (N=7), respectively. Although there is an apparent difference between mean EM of *Aam*Ach-S/L-dsRNA (441.1±92.27 mg) and GFP-dsRNA (504.2±53.66 mg) injected ticks (not shown), Student's unpaired t-test showed that differences were not statistically significant (P=0.5656). To determine the effect of RNAi-mediated silencing on fecundity, ticks were allowed to oviposit for 30 days and egg clutches were weighed. As an index of the tick's ability to convert its blood meal to eggs, the egg mass conversion ratio (EMCR) was calculated by dividing the egg weight (not shown) by EM. Calculated EMCR for GFP-dsRNA and *Aam*Ach-S/L-dsRNA injected ticks ranged from 594.9 to 449.1 mg (N=3) and 532.7 to 258.2 mg (N=3), respectively. While the EMCR of *Aam*Ach-S/L-dsRNA (417.0±82.12 mg) injected ticks is apparently smaller than GFP-dsRNA injected ticks (539.3±45.52 mg) (not shown), the observed difference is not statistically significant as revealed by Student's unpaired t-test (P=0.2626). Please note that, although seven spontaneously detached ticks were incubated to lay eggs from each treatment group, only three ticks each from control and treatment groups were able to oviposit, and the remaining four ticks did not.

DISCUSSION

A putative acidic chitinase-like encoding cDNA fragment was found among feeding stimuli responsive *A. americanum* genes (Mulenga et al., 2007). In this study we show that *A. americanum* constitutively and ubiquitously expresses the long and short putative acidic chitinase (Ach) forms. *Aam*Ach-L and *Aam*Ach-S were provisionally identified as members of the GH-18 family based on amino acid identity to previously annotated GH-18 chitinases, and conservation of amino acid motifs that characterize the GH-18 family (Arakane and Muthukrishnan, 2010). Based on data mining in this study, the tick GH18-like chitinases are a multi-member family. The observation that with exception of *R. sanguineus* GH-18 chitinase sequence (ACX33152.1) that showed >80 amino acid identity, *Aam*Ach-L and *Aam*Ach-S amino acid identities to other tick GH-18 chitinase sequences were ≤50% may suggest that tick GH-18 chitinases regulate multiple non-redundant pathways. At the time of this write-up genome sequence data for many tick species was not available, with exception of *I. scapularis*, and thus the comparative sequence and phylogeny analyses presented here may be incomplete.

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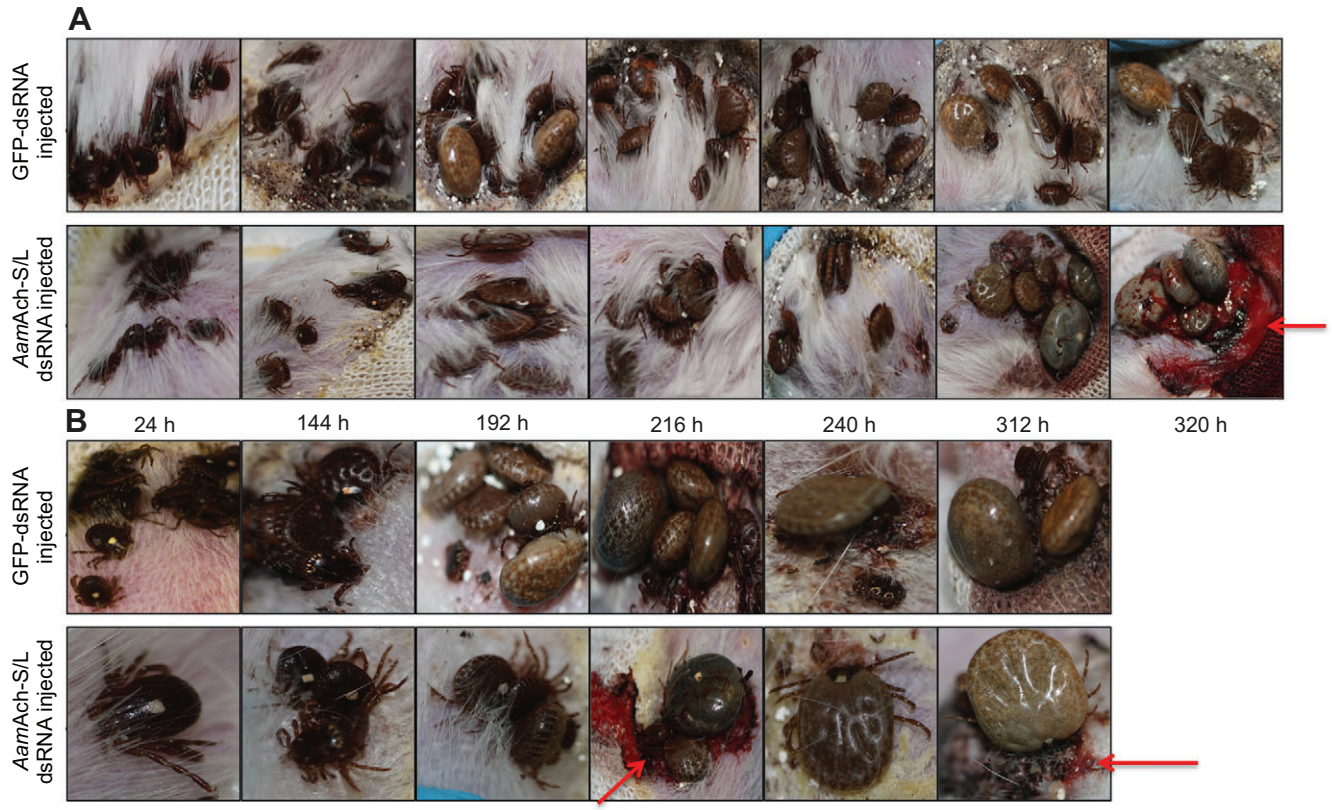

Fig. 9. Phenotype of *Aam*Ach-S/L dsRNA injected ticks. Ticks were photographed starting from 24 h post-attachment until the end of the experiment to document changes during tick feeding in *Aam*Ach-S/L-dsRNA injected compared with control GFP-dsRNA injected ticks. The experiment was repeated twice: (A) set of pictures from first trial that was stopped at 320 h post-attachment due to excessive bleeding from host at tick feeding site and (B) set of pictures from second trial where ticks completed feeding. The red arrows indicate the excess pool of blood around the feeding site, not observed in control GFP-dsRNA injected on both trials.

Expression of *Aam*Ach-L and *Aam*Ach-S mRNA in multiple tick organs observed in this study signals the possibility that native *Aam*Ach-L and *Aam*Ach-S proteins could be important to tick physiology in general. Our quantitative RT-PCR analysis data are suggestive of the possibility that *Aam*Ach-L proteins could be associated with tick feeding physiology at the salivary gland level, and not at the mid-gut level. In tick research, genes that are up-regulated in response to feeding are thought to be associated with blood meal feeding (Mulenga et al., 2007; Aljamali et al., 2009; Mulenga and Khumthong, 2010; Konnai et al., 2011) and those that are down-regulated are believed to play other roles in tick biology, and are not associated with blood meal feeding (Umemiya et al., 2008; Aljamali et al., 2009). Given that *Aam*Ach-L mRNA abundance in the SG did not apparently change with feeding could signal the importance of this protein in unfed and during the parasitic stages of *A. americanum*. We were unable to quantitatively determine *Aam*Ach-S expression levels, and the relationship of *Aam*Ach-S to the tick feeding cycle remains unknown.

The observation in this study that dual silencing affected the stability of *A. americanum* tick cement was significant. Seepage of blood around mouthparts, and the fact that ticks easily detached with a light touch was suggestive of malformed or weakened tick cement cone, thus suggesting that *Aam*Ach-L and/or *Aam*Ach-S were important to tick feeding success. Within 5–30 min of attaching onto the host, a tick secretes an amorphous adhesive substance called cement to anchor itself onto host skin (Sonenshine, 1993). Secretion of tick cement is by far one of the most significant biological adaptations that make ticks successful as pests and vectors. Without tick cement, hard ticks will easily be groomed off the host before they complete feeding, acquiring and/or transmitting disease agents. Hard ticks are long-term blood feeders that remain securely attached onto host skin for long periods of time, 4–7 days for larva and nymphs, and 10–14 days for adult ticks with the help of an intact cement plug (Sonenshine, 1993). Without the cement cone, ticks can be easily groomed off the host. The compositions of tick cement or molecular cascades that lead to tick cement formation remain unknown. Thus the finding that *Aam*Ach-S/L could be associated with tick cement stability advances our knowledge in this regard. Finding ways to disrupt tick cement is an attractive target for development of novel tick control methods. It is important to note here that leakage of blood was not observed in all *Aam*Ach-S/L-dsRNA injected ticks. We are of the opinion that this could be explained by the possibility that ticks did not receive the same *Aam*Ach-S/L-dsRNA dosage. This is supported by our RNAi silencing validation data that show that *Aam*Ach-S/L mRNA was disrupted to different levels in different tick individuals.

Another notable observation in this study is that the observed effect of silencing is relatable to temporal and spatial transcription of *Aam*Ach-L. Bleeding around mouthparts in *Aam*Ach-S/L-dsRNA injected ticks was observed at day 13, late in the feeding cycle. Interestingly, transcription analysis show an initial decrease in *Aam*Ach-L transcript abundance during the first 3 days of feeding, before it increased, albeit not statistically significant in salivary gland and other tissues. Based on these observations it is logical to speculate that *Aam*Ach-L and potentially *Aam*Ach-S are functionally important late during the tick feeding process. It is also interesting

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to note that four of the seven ticks that were incubated to lay eggs did not oviposit for both GFP and *Aam*Ach-S/L-dsRNA injected ticks. We would like to note here that our injection site was right behind the fourth leg coxa. The tick ovary loops around the body near to our injection site, and thus there is potential that we caused traumatic injury to ovaries. This could have affected egg development.

We are interested in understanding the role(s) of tick saliva proteins in tick feeding physiology. Thus we wanted to functionally characterize r*Aam*Ach-L, which is predicted to be extracellular. Consistent with bioinformatics prediction of multiple putative *N*- and *O*-linked glycosylation sites, r*Aam*Ach-L was significantly glycosylated. This meant that r*Aam*Ach-L was potentially well folded with appropriate post-translational modifications, and thus functional analysis data could potentially reflect *in vivo* events. Data in this study suggest that native *Aam*Ach-L is potentially an inactive GH-18 chitinase. Within the GH-18 family functional domain amino acid motif 'FDG(L/F)DL**D**W**E**(Y/F)P', aspartic acid (D) and glutamic acid (E) (highlighted in bold) amino acid residues were thought to be important in activity of GH-18 chitinases (Arakane and Muthukrishnan, 2010). It is notable that in *Aam*Ach-L predicted functional domain (LDGVDM**A**W**P**FP), 'D' is replaced by 'A', while 'E' is replaced by 'P'. Further investigations are needed to determine if these mutations are indeed important for *Aam*Ach-L function. The observation here is consistent with reported studies where GH-18 chitinase-like proteins in *Tribolium castaneum*, *Autographa californica* and *Manduca sexta* that have mutations similar to *Aam*Ach-L were not functional chitinases (Thomas et al., 2000; Lu et al., 2002; Arakane and Muthukrishnan, 2010). It is also important to note that *H. longicornis* tick chitinase (accession number: BAC06447.1) reported in the phylogeny analysis retains amino acid residues that are important for GH-18 chitinase function (You et al., 2003). It is interesting to note that all the sequences within cluster B and C contained aspartic acid (D) and glutamic acid (E) for the functional amino acid motif FDG(L/F)DL**D**W**E**(Y/F)P (not shown). However, sequences in cluster A did not retain the aspartic acid (D) and glutamic acid (E). This may imply that ticks encode both a functional and non-functional GH-18-like chitinases. It is also interesting to note that both *Aam*Ach-L and *Aam*Ach-S do not have putative chitin-binding domains, which are thought to bind chitin prior to its degradation. The absence of the domain does not affect the ability of the enzyme to hydrolyse the soluble substrate triacetylchitotriose, but abolishes its ability to hydrolyse insoluble chitin (Tjoelker et al., 2000). Whether or not the absence of these sequence features caused the lack of chitinase activity in r*Aam*Ach-L remains to be investigated.

Our western blotting analysis experiments to validate if native *Aam*Ach-L was immunogenic and injected into the host during tick feeding was inconclusive, in that we observed that r*Aam*Ach-L bound both pre-immune and tick saliva protein immune sera. Interestingly, in an ongoing study in our group, we have found a putative acidic chitinase in an *I. scapularis* tick that shows 61% amino acid identity to *Aam*Ach-L (T.K.K., L. Tirloni and A.M., unpublished observations) and have verified the presence of *Aam*Ach-L in unfed and fed *A. americanum* tick saliva proteomes (T.K.K., L. Tirloni and A.M., unpublished observations). Additionally in a recent study, Tirloni et al. (Tirloni et al., 2014) found a putative acidic chitinase that shows 79% amino acid identity to *Aam*Ach-L in saliva of *R. microplus*. Based on these observations we have concluded that *Aam*Ach-L is a tick saliva protein that may be part of the tick's system to eliminate host immunoglobulins during feeding. There is precedence in *R. appendiculatus* where host antibody elimination was documented (Wang and Nuttall, 1994).

In conclusion this research has made a contribution towards understanding the molecular basis of the tick attachment phase. This research has opened up opportunities to further investigate the role(s) of putative *Aam*Ach-L and *Aam*Ach-S in maintaining the stability of the tick cement cone. Important questions that remain to be resolved are if *Aam*Ach-L is part of the tick machinery to eliminate host antibodies, and if it is part of the cement cone or associated with upstream tick cement formation cascades. Given the apparent importance in maintenance of tick cement cone stability it will be interesting to investigate if this protein or its interacting molecular partners can be targeted for tick control. Based on data mining in this study, it is also apparent that the tick GH-18 family has multiple members. Thus to understand the role(s) of the GH-18 family in tick physiology, these genes must be investigated as a group.

MATERIALS AND METHODS

Tick feeding, dissections, total RNA extractions and cDNA synthesis

Amblyomma americanum ticks were purchased from tick laboratories at Texas A&M University and Oklahoma State University (College Station, TX, USA and Stillwater, OK, USA, respectively). Routinely, ticks were fed on rabbits according to animal use protocols approved by Texas A&M University Institutional Animal Care and Use Committee. To feed, *A. americanum* ticks were placed onto the outer part of the ear of specific pathogen-free (SPF) New Zealand rabbits. Ticks were restricted onto the outer ears using orthopedic stockinette glued onto the rabbit ear with Kamar Adhesive (Kamar Products Inc., Zionsville, IN, USA). Six male ticks were pre-fed for 3 days prior to introducing 15 female ticks on each ear stocking (total of 30 female ticks per rabbit).

Two rounds of dissections were done to produce material for qualitative and quantitative RT-PCR analysis. In the first round of dissections to prepare material for qualitative RT-PCR, five ticks per time point were sampled at 24, 48, 72, 96 and 120 h. Tick organs including salivary glands (SG), midguts (MG), ovary (OV), synganglion (SYN) Malpighian tubules (MT) and carcass (CA, the remnants after removal of other organs) were isolated and placed in 1 ml of the RNA extraction solution, Trizol (Life Technologies, Carlsbad, CA, USA) and stored at −80°C until total RNA extraction. In the second round of dissections to prepare material for quantitative RT-PCR analysis, triplicate pools of 10, eight and five ticks were dissected at the unfed and 24, 48−96 and 120 h fed time points, respectively. Tick organs SG, MG and CA were pooled in 1 ml Trizol and stored at −80°C until total RNA extraction. Within the first hour of detachment, ticks were prepared for dissections as described by Mulenga et al. (Mulenga et al., 2013). Prior to dissecting, tick mouthparts were inspected to remove any remaining rabbit tissue and washed in RNase inhibitor diethylpyrocarbonate (DEPC)-treated water.

Total RNA was extracted using the Trizol reagent according to the manufacturer's instructions (Life Technologies), resuspended in DEPC-treated water, and quantified using a UV-VIS Spectrophotometer DU-640B (Beckman Coulter, Brea, CA, USA). Total RNA (1 µg) was used to synthesize cDNA using the Verso cDNA Synthesis Kit following the manufacturer's instructions (Thermo Scientific, Waltham, MA, USA). The synthesized cDNA was quantified as above and stored at −20°C until use.

Full-length *A. americanum* Ach cDNA cloning, bioinformatics and phylogeny analyses

The full-length cDNA sequence of putative *Aam*Ach cDNA was cloned using the RACE technique (ClonTech, Mountain View, CA, USA) according to the manufacturer's protocol using 120 fed tick cDNA. PCR fragments were routinely cloned into the pGEM-T TA cloning vector (Promega, Madison, WI, USA) and sequenced with T7 and SP6 promoter primers. Sequences were analysed using the MacVector program (MacVector Inc., Cary, NC, USA).

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Validating if *A. americanum* expresses long and short *Aam*Ach forms

Preliminary sequence analysis identified *Aam*Ach-S with a 210 bp nucleotide deletion between position 817−1026 bp of *Aam*Ach-L. To determine if both *Aam*Ach-L and *Aam*Ach-S mRNAs were expressed, respective specific forward 5′-GAGGGGAGTCTGGAACGGAG-3′ and 5′-CGCCCAAGCCCACGCCCTCGTC-3′ and the universal reverse 5′-GAAGTCGTCCAGGTCGTTGTTG-3′ PCR primers were designed as illustrated in Fig. 1A. The *Aam*Ach-S specific forward primer overlapped the deletion site, while the *Aam*Ach-L forward primer was designed inside the deletion site.

Bioinformatics and phylogeny analyses

BlastX and BlastP homology scanning of *Aam*Ach-L and *Aam*Ach-S at the National Center for Biotechnology Information and UniProt were used to identify protein sequences in other organisms that showed identity to *Aam*Ach. Scanning on SignalP version 4.1 (Petersen et al., 2011) and NetNGlyc 1.0 and NetOGlyc 4.0 (Steentoft et al., 2013) servers detected presence of a signal peptide and putative *N*- and *O*-linked glycosylation sites, while scanning in GenBank and visual inspection of motifs described in Arakane and Muthukrishnan (Arakane and Muthukrishnan, 2010) detected annotated amino acid motifs. Calculated molecular weights were determined using the MacVector (MacVector Inc.) protein toolbox.

Tick GH-18-like chitinases in public databases GenBank (*N*=5) and UniProt (*N*=5) sequence were downloaded and used to construct a guide phylogeny tree using MEGA 5.2.2 online software (http://www.megasoftware.net). Based on preliminary blast results, *Aam*Ach-L and *Aam*Ach-S were categorized as a GH-18 chitinase-like protein, therefore *Homo sapiens* (accession number: AAA35684.1) GH-18 chitobiase was used as an out-group in the phylogeny tree. Sequences were aligned using ClustalW of ~350 amino acid conserved cysteine amino acid residue region $C_{70–417}$ and the phylogeny tree constructed using the maximum likelihood method using the Whelan and Goldman (WAG) model option (Whelan and Goldman, 2001). To estimate bootstrap values, replications were set to 1000.

Temporal and spatial qualitative and quantitative RT-PCR transcription analyses

Qualitative and quantitative RT-PCR analyses determined temporal and spatial mRNA profiles. In qualitative RT-PCR, *Aam*Ach-L and *Aam*Ach-S specific PCR primers (Fig. 1A) were used with cDNA of 24, 48, 72, 96 and 120 h fed tick dissected SG, MG, OV, SYN and CA. For both putative *Aam*Ach-L and *Aam*Ach-S, PCR cycling conditions were an initial denaturing step at 94°C followed by 30 amplification cycles of 94°C for 30 s, 53°C for 30 s, 72°C for 1 min, and a final extension step of 72°C for 5 min. PCR products were electrophoresed on 2% agarose gels containing 1 µg ml^{-1}.

In quantitative RT-PCR analysis triplicate pools of unfed, 24, 48, 72, 96 and 120 h tick SG, MG and CA were analysed using an Applied Biosystems 7300 Real Time PCR System (Life Technologies) using specific *Aam*Ach-L forward (5′-CTGGAACGGAGTTACCGATGTGC-3′) and reverse (5′-TGCGGACCGATATCAATGGAT-3′) qRT-PCR primers. In preliminary experiments efforts to find optimal qRT-PCR primers for *Aam*Ach-S failed, and thus this analysis was abandoned. Reaction volumes in triplicate contained 10-fold diluted cDNAs that were originally synthesized from 1 µg total RNA, 350 nmol l^{-1} of forward and reverse *Aam*Ach-L primers, and 2X SYBR Green Master Mix (Life Technologies). For internal control, forward (5′-GGCGCCGAGGTGAAGAA-3′) and reverse (5′-CCTTGCCGTCCA-CCTTGAT-3′) primers targeting a 55 amplicon of *A. americanum* 40S ribosomal protein S4 (RPS4; accession number: GAGD01011247.1) that is stably expressed in *I. scapularis* during feeding (Koči et al., 2013) was used.

Relative quantification (RQ) of *Aam*Ach-L transcript was determined using the comparative C_T ($2^{-\Delta\Delta C_T}$) method (Livak and Schmittgen, 2001) on a Microsoft Excel spreadsheet (Microsoft Corporation, Redmond, WA, USA). Based on the C_T values of the three biological replicates for each dissected tissues, the ΔC_T values were determined by the formula C_T (*Aam*Ach) – C_T (RPS4) per time point. Subsequently, the $\Delta\Delta C_T$ values were determined using the formula ΔC_T (replicate) – C_T (average of replicates per tissue) per time point for each individual replicate. The fold change was determined for each replicate in each tissue by using the formula $2^{-\Delta\Delta CT}$ and plotted on an Excel spreadsheet to generate a chart. Statistical analyses were performed using non-parametric Student's *t*-tests with Prism software (GraphPad Software, San Diego, CA, USA).

Expression and affinity purification of r*Aam*Ach-L

Recombinant *Aam*Ach-L protein was expressed using the *P. pastoris* and pPICZα plasmid expression system (Life Technologies). The expression plasmid was constructed by subcloning the mature *Aam*Ach-L coding domain into pPICZαA *Kpn*I and *Not*I sites, using forward (5′-**GGTACC**ATGCCCCAGCAAGACGGGGATG-3′) and reverse (5′-**GCGGCCGC**AGCGGGTGATGCGGTAATCTCG-3′) primers with added restriction enzyme sites in bold. The pPICZαA-*Aam*Ach-L expression plasmid was linearized with *PmeI* and used to transform *P. pastoris* X-33 strain (Life Technologies) by electroporation as described previously (Mulenga et al., 2013). Likewise, induction, validation and affinity purification of recombinant protein expression were done as described previously (Mulenga et al., 2013). Affinity purified putative r*Aam*Ach-L was dialysed against 1×phosphate-buffered saline (PBS) at pH 7.4 for downstream assays. Routinely, affinity purified r*Aam*Ach-L was resolved on a 10% SDS-PAGE gel and silver stained to verify purity and background contamination. Samples with least background were selected and concentrated by either ammonium sulphate precipitation or by centrifugation using MicroSep Centrifugal Concentration Devices (Pall Corporation).

N- and *O*-linked deglycosylation assay

To determine if r*Aam*Ach-L was *N*-glycosylated and/or *O*-glycosylated, 2.5 µmol l^{-1} affinity purified r*Aam*Ach-L was treated with protein deglycosylation enzyme mix according to the manufacturer's instructions (New England Biolabs, Ipswich, MA, USA). Deglycosylation was verified by western blotting analysis using the antibody to c-terminus hexa histidine-tag (Life Technologies) and the positive signal detected using horseradish peroxidase (HRP) chromogenic substrate (Thermo Scientific).

Determining if native *Aam*Ach-L is injected into the host during tick feeding

To determine if native *Aam*Ach-L is injected into the host during feeding, affinity purified r*Aam*Ach-L was subjected to routine western blotting analyses using antibodies to 48 h and replete fed *A. americanum* tick saliva proteins. Antibodies used here were produced by repeated tick infestation of rabbits every 48 h (Chalaire et al., 2011) and repeatedly allowing ticks to feed to repletion (Mulenga et al., 2013). Our preliminary analysis showed that r*Aam*Ach-L non-specifically reacted with both pre-immune (PI) serum and immune sera to tick saliva proteins. To validate if r*Aam*Ach-L non-specifically binds immunoglobulins, deglycosylated and glycosylated r*Aam*Ach-L was further subjected to western blot analyses using PI sera of chicken and bovine. In all western blotting analyses, 1:2000 antibody dilutions were used.

Substrate hydrolysis assay

Chitinase function assays were done by substrate hydrolysis using a commercial kit from Sigma (St Louis, MO, USA; catalogue no. CS0980). Substrates used were nitrophenyl *N*,*N*′-diacetyl-β-D-chitobioside and 4-nitrophenyl *N*-acetyl-β-D-glucosaminide for exochitinase activity and 4-nitrophenyl β-D-*N*,*N*′,*N*″-triacetylchitotriose substrate for endochitinase activity. Prior to beginning the assay, standard and substrate solutions were equilibrated to 37°C by incubating for 10 min in a 37°C water bath. The pre-warmed substrates 50 µl (1 mg ml^{-1}) were mixed with r*Aam*Ach-L (1.91 and 0.477 µmol l^{-1}), and positive (0.8 and 0.2 µmol l^{-1}) or negative (1.91 and 0.477 µmol l^{-1}) controls into a 96-well plate. Validated *T. viride* active chitinase was used as a positive control. The standard reaction was placed into separate wells on the plate and the whole plate was mixed by shaking in the plate reader and incubated for 30 min at 37°C. The reaction was stopped by addition of 100 µl of stop solution (0.4 mol l^{-1} sodium carbonate) and the end-point was measured at A_{405} using the Infinite M200 Pro plate reader (Tecan, Männedorf, Switzerland) pre-warmed to 37°C. The experiment was also repeated using different mixtures of 0.1 mol l^{-1} citric

acid (A) and 0.2 mol l^{-1} dibasic phosphate (B) buffer at pH values of 4 (61.45 ml A plus 38.55 ml B), 5 (48.5 ml A plus 51.5 ml B), 5.4 (44.25 ml A plus 55.75 ml B) and 6 (36.85 ml A plus 63.15 ml B) as described by Carmody (Carmody, 1963).

RNA interference (RNAi) mediated *Aam*Ach silencing

RNAi-mediated silencing was performed as previously published (Mulenga et. al., 2008). dsRNA targeting both *Aam*Ach-L and *Aam*Ach-S was synthesized *in vitro* using the Megascript RNAi kit (Life Technologies) according to the manufacturer's protocol. A 466 bp cDNA fragment from position 155–620 of *Aam*Ach-L and 136–602 *Aam*Ach-S cDNA sequences (see Fig. 7) was used for *Aam*Ach-S/L-dsRNA synthesis. Templates for dsRNA synthesis were amplified using cloned *Aam*Ach-L plasmid DNA using specific primers with added T7 promoter sequence in bold (forward: 5′-**TAATACGACTCACTATAGGG**CCACGTCAAGCACCACCACC-3′ and reverse: 5′-**TAATACGACTCACTATAGGG**GCGTAGTTTTCCTT-ATCGCG-3′). Enhanced green fluorescent protein coding cDNA (EGFP; accession number: JQ064510.1) was amplified as described previously (Mulenga et al., 2008). Two test groups of 15 female *A. americanum* ticks were injected with GFP-dsRNA or *Aam*Ach-S/L-dsRNA. Ticks were injected with 0.5–1 µl (~3 µg $µl^{-1}$) of dsRNA on the ventral side of the lower right coxa using a 33-gauge/0.5 inches/45 deg angle bevelled needle using a model 701 Hamilton syringe (Hamilton Company, Reno, NV, USA). Injected ticks were kept for 24 h at room temperature in 85% humidity to recover before being placed on SPF New Zealand rabbits to feed.

Ticks injected with *Aam*Ach-S/L-dsRNA (N=15) and GFP-dsRNA (N=15) were fed on rabbits as described above. Effect of RNAi silencing on tick feeding success was assessed by investigating tick attachment and mortality rates, time to feed to repletion, EM as an index for amount of blood taken in by tick, and EMCR as a measure of utilizing blood meal to produce eggs. Attachment rates were determined by daily counts of unattached ticks, and then subtracting from the total number of ticks that were placed on the animal less those that were found dead. Mortality rates were determined by calculating dead ticks as a fraction of the total number of ticks. EM was the weight of spontaneously detached ticks. To determine EMCR, ticks were allowed to lay eggs for 3–4 weeks at room temperature in 85% humidity. EMCR was determined by dividing egg mass by EM. Tick phenotypes during feeding were documented daily using the Canon EOS Rebel XS camera attached to a Canon Ultrasonic EF 100 mm 1:2:8 USM Macro Lens (Canon USA Inc., Melville, NY, USA). This was repeated one more time.

Validation of RNAi silencing

To validate if injection of dsRNA caused disruption of *Aam*Ach-S/L mRNA, three ticks each of GFP dsRNA and *Aam*Ach dsRNA injected females were sampled at 48 h post-attachment by manual detachment. Ticks were processed individually for dissection of tick organs as described above. Dissected organs, SG, MG, SYN, MT, OV and remnants labelled as CA, preserved in 200–400 µl RNAlater (Life Technologies), were processed for mRNA extraction using the Dynabead mRNA Direct Kit (Life Technologies). Concentration of mRNA was determined using the NanoQuant Plate in the Infinite M200 Pro plate reader (Tecan) and template cDNA synthesized from ~200 ng of mRNA using the qScript cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD, USA).

Validating of RNAi silencing by qRT-PCR was done as described above. Approximately 50 ng or 20-fold of cDNA, and 900 nmol l^{-1} of *Aam*Ach-L forward 5′-GGGAACTGGCTGGTGCATT-3′ and reverse 5′-GAGCGG-CACGACGACTGT-3′ primers were added to 2×SYBR Green Master Mix (Life Technologies) in triplicate. To determine the apparent level of *Aam*Ach mRNA suppression, the formula $S = 100 - (RQ^{T}/RQ^{C} \times 100)$ was used, where S is mRNA suppression, and RQ^{T} and RQ^{C} are RQ of tissues in *Aam*Ach-S/L-dsRNA and GFP-dsRNA injected ticks, respectively. For each tick organ, mRNA suppression was determined as the mean ± s.d. of two S values. The two S values were generated by independently comparing treatment RQ to two separate control tick RQs.

Statistical analysis

To investigate if observations were significantly different between treatments, in the substrate hydrolysis, effects of RNAi silencing on tick feeding and validation of RNAi silencing analyses were subjected to non-parametric Student's t-test set to 95% confidence interval. One-way ANOVA and Tukey's honestly significant difference test were used to determine if differences in transcript abundance between unfed ticks and ticks fed for 24, 48, 72, 96 and 120 h were statistically significant. The statistical software package Prism version 6 (GraphPad Software Inc.) was used.

Competing interests

The authors declare no competing financial interests.

Author contributions

T.K.K. and A.M. participated in the experimental design, data analysis, interpretation and write up; T.K.K. and J.C. collected data.

Funding

This work was supported in part by start-up funds from Texas A&M University and research grants from the National Institute of Allergy and Infectious Diseases/National Institutes of Health (NIAID/NIH, AI081093 and AI1AI093858) to A.M. J.C. was a 2008 cohort REU-EXCITE program student. Deposited in PMC for release after 12 months.

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