The Journal of Experimental Biology 210, 3919-3930
Published by The Company of Biologists 2007
doi:10.1242/jeb.008342

Cloning and characterization of AgCA9, a novel α-carbonic anhydrase from *Anopheles gambiae* Giles *sensu stricto* (Diptera: Culicidae) larvae

Kristin E. Smith, Leslie A. VanEkeris and Paul J. Linser*

The Whitney Laboratory for Marine Biology, University of Florida, 9505 Ocean Shore Boulevard, Saint Augustine, FL 32080, USA

*Author for correspondence (e-mail: pjl@whitney.ufl.edu)

Accepted 16 August 2007

Summary

Mosquito larvae generate a luminal pH as high as 10.5 in the anterior region of their midgut. The mechanisms responsible for the generation and maintenance of this alkaline pH are largely unknown, but there is evidence suggesting a role for the enzyme carbonic anhydrase (CA). CA has been cloned from the alimentary canal epithelium of *Anopheles gambiae* larvae and can generate bicarbonate, which is implicated as a buffer for the larval lumen. The question remains as to how the bicarbonate is transported from the cells into the lumen. We hypothesize the presence of a CA within the lumen itself to generate bicarbonate from CO_2 produced by the metabolically active alimentary canal cells. Here, we report the cloning and characterization of a novel cytoplasmic-type α-CA from the larval *An. gambiae* alimentary canal. Antibody immunolocalization reveals a unique protein distribution pattern that includes the ectoperitrophic fluid, 'transitional region' of the alimentary canal, Malpighian tubules and a subset of cells in the dorsal anterior region of the rectum. Localization of this CA within the lumen of the alimentary canal may be a key to larval pH regulation, while detection within the rectum reveals a novel subset of cells in *An. gambiae* not described to date. Phylogenetic analysis of members of the α-CA family from the *Homo sapiens*, *Drosophila melanogaster*, *Aedes aegypti* and *An. gambiae* genomes shows a clustering of the novel CA with *Homo sapiens* CAs but not with other insect CAs. Finally, a universal system for naming newly cloned *An. gambiae* CAs is suggested.

Key words: carbonic anhydrase, mosquito larvae, ectoperitrophic space, rectum, pH regulation, midgut alkalization.

Introduction

Mosquito larvae, like caterpillars, generate a luminal pH in excess of 10.5, one of the highest pH values known in any biological system (Dadd, 1975). The use of this high pH to initiate digestion sharply contrasts most other organisms, which initiate digestion in conditions that are more acidic and can reach a pH equivalent to battery acid (~pH 1.0) in certain situations. This high pH is thought to have a role in the dissociation of tannin–protein complexes that are found in the phytophagous diet of caterpillars and other insect larvae (Berenbaum, 1980).

The larval alimentary canal can be divided into six main sections: gastric caeca (GC), anterior midgut (AMG), posterior midgut (PMG), Malpighian tubules (MT), ileum and rectum. The highly alkaline pH is restricted to the AMG lumen and exists in the absence of any morphological barriers between it and the near-neutral pH values of the GC and PMG lumina. Currently, a detailed understanding of the mechanisms driving digestion and pH/ion regulation in these regions of the alimentary canal is lacking; however, there is evidence supporting a role for the enzyme carbonic anhydrase (CA) (Corena et al., 2002; Seron et al., 2004) (T. J. Seron, personal communication).

Multiple CAs have been cloned from, and immunolocalized to, the *Anopheles gambiae* (Seron et al., 2004) (T. J. Seron, personal communication; K.E.S., unpublished data) and *Aedes aegypti* (Corena et al., 2002; Seron et al., 2004) midguts and it has been shown that inhibition of CA in mosquito larvae blocks alkalization (Corena et al., 2002). CA catalyzes the reversible conversion of $H_2O+CO_2 \rightleftarrows H^+ + HCO_3^-$ and can generate HCO_3^- (bicarbonate) within midgut epithelial cells. Living larvae actively excrete bicarbonate, resulting in a net alkalization of their surrounding media (Stobbart, 1971). Moreover, addition of the global CA inhibitor, methazolamide, inhibits this alkalization (Corena et al., 2002). These data implicate CA as a key player in midgut alkalization.

A current view of larval midgut alkalization assumes that CA in the epithelial cells catalyzes the formation of bicarbonate, which is then translocated into the lumen by an as yet unidentified plasma membrane anion exchanger. Bicarbonate can then be deprotonated to carbonate (pK_{a_2}=10.32) (Dow, 1984). Together with a strong cation such as a potassium or sodium ion, carbonate could serve as the buffer in the highly alkaline AMG lumen. Despite numerous attempts, an anion exchanger has not been detected in the apical membrane of the midgut epithelium. This anion exchanger is necessary for the translocation of bicarbonate from the epithelial cells into the lumen, where it is needed to buffer the alkaline contents. We

hypothesize an alternative mechanism for supplying the lumen with bicarbonate: an extracellular CA within the actual lumen of the alimentary canal may obviate the need for membrane anion exchange and would supply the lumen directly with bicarbonate, as will be described in this paper.

Here, we report the cloning and characterization of a novel cytosolic-like α-CA (GenBank accession no. DQ518576), henceforth referred to as AgCA9, from the alimentary canal of *An. gambiae* mosquito larvae. The protein has been localized to the ectoperitrophic fluid, the epithelial cells in the region where the alimentary canal transitions from the AMG to the PMG (transitional region; TR) (Clark et al., 2005), the principal cells of the MT, and the cells of the dorsal anterior region of the rectum. A CA within the ectoperitrophic space of the alimentary canal could be involved in the production of a constant supply of bicarbonate within the lumen, the ion thought to regulate alkalization. Additionally, the localization of this protein to a subset of cells in the rectum challenges an established paradigm of mosquito cell biology. The rectum of freshwater mosquitoes has traditionally been described as being uniform in function and structure (Meredith and Phillips, 1973). The differential expression of this CA raises the question of whether the rectum of the freshwater breeder, *An. gambiae*, differs from this model by having two distinct regions. This would be the first description of a freshwater mosquito larva with this characteristic. We report the phylogenetic analysis of members of the α-CA family from the *Homo sapiens*, *Drosophila melanogaster*, *Ae. aegypti* and *An. gambiae* genomes and show that AgCA9 clusters with *H. sapiens* cytoplasmic CAs. We also suggest a uniform naming system for newly cloned *An. gambiae* CAs, which to date does not exist.

Materials and methods

Model organism

Anopheles gambiae SS G3 strain larvae were hatched from eggs supplied by MR4 (The Malaria Research and Reference Reagents Resource Center) at the Centers for Disease Control and Prevention in Atlanta, GA, USA (http://www.malaria.atcc.org) and raised to the 3rd–4th instar as described in the supplier manual (www2.ncid.cdc.gov/vector/vector.html) under a 12 h:12 h dark:light interval and 2-day feeding schedule.

Cloning of AgCA9

Primers were designed to an internal region of AgCA9 (ENSANGG00000016723) as predicted by ensembl (www.ensembl.org): forward primer, GGGATACACGCAA-ATGAAC; reverse primer, GCTATCGACTTCACGCAG. These primers were used in a PCR reaction to amplify the CA from cDNA collections made to include the entire larval alimentary canal. cDNA collections were generated as described in Matz, (Matz, 2003). Once amplified, the PCR product was sequenced using the ABI Prism Big Dye Terminator Cycle Sequencing Kit (PE Biosystems, Foster City, CA, USA), and the reaction products were analyzed on an ABI Prism 310 Genetic Analyzer. This known sequence was used to design gene-specific primers for the rapid amplification of cDNA ends (RACE). RACE was used to determine the 3′ and 5′ ends (Zhang and Frohman, 1997) [modified by Matz et al. (Matz et al., 1999)] using cDNA generated as described above. Once the full-length sequence was obtained, gene-specific primers were designed to amplify the full-length AgCA9 DNA. Forward primer, ATGTCT-GTCACTTGGGGATACAC; reverse primer, TTAGTAGC-TATCGACTTCACGCAG. The full-length gene was ligated into pCR4-TOPO (Invitrogen, Carlsbad, CA, USA) and transformed into Top10 chemically competent bacterial cells (Invitrogen) for sequence confirmation as described above.

Quantitative PCR (QPCR)

RNA was extracted (TRI reagent®–RNA/DNA/Protein isolation reagent; Molecular Research Center, Inc., Cincinnati, OH, USA) from whole alimentary canal, GC, AMG, PMG, MT and rectum from early 4th-instar *An. gambiae* mosquito larvae. Superscript II Reverse Transcriptase kit (Invitrogen) was used to generate cDNA from RNA using manufacturer's instructions. Primers were designed to both AgCA9 and an 18s ribosomal RNA endogenous control using ABI primer express software. AgCA9 forward primer, GAGCTTTTCCGCGAAATGC; AgCA9 reverse primer, CTCGTCGCAGGGACACTCTT. 18s forward primer, GCGACCTCGTCGGTCAAG; 18s reverse primer, ATGGTGCCCGGGAACTCT. Primers were not designed to span an intron, as is generally suggested to minimize genomic DNA contamination, but RNA samples that were treated with DNAseI to eliminate genomic DNA produced identical results. QPCR was performed using the Applied Biosystems (ABI, Foster City, CA, USA) 7000 Sequence Detection system, and data were analyzed using the method described in Pfaffl (Pfaffl, 2001). Each reaction was run in triplicate.

In situ hybridization

Digitoxin-labeled RNA probes were designed to the full-length AgCA9 using the DIG RNA labeling kit (Roche, Nutely, NJ, USA). Ten early 4th-instar *An. gambiae* midguts were dissected, five for sense and five for antisense detection. To dissect the gut, the heads of the cold-immobilized larvae were pinned down using fine stainless-steel pins to a Sylgard layer at the bottom of a Petri dish containing 4% paraformaldehyde in phosphate-buffered saline (PBS). The anal segment and the saddle papillae were removed using ultra-fine scissors and forceps, and an incision was made longitudinally along the thorax. The carcass, including the fat body, central nervous system, trachea and muscle, was separated from the gut. AgCA9 RNA was detected according to the *in situ* hybridization protocol outlined in Meleshkevitch et al. (Meleshkevitch et al., 2006).

Antibody production

Chicken antibodies against AgCA9 were generated by Aves Labs, Inc. (Tigard, OR, USA) for use in western blotting and immunohistochemistry. Antibody 5340 was generated against the BSA-conjugated peptide CZELGNRQLREVDSY and was used as purified IgY. Additionally, antibody 5563 was generated against the BSA-conjugated peptide KEPIEVSHE-QLELFREMRC and was affinity purified before use using the immunogen peptide.

To localize Na^+/K^+-ATPase, monoclonal antibodies that had been raised against the α-subunit of avian P-type Na^+/K^+-

ATPase in mice were obtained from the Developmental Studies Hybridoma Bank (Lebovitz et al., 1989).

Blocking assay

To determine specificity, antibody was pre-incubated with peptide prior to detection of protein. First, the amount of antibody to be used in milligrams (mg) was calculated based on a 1:1000 dilution. Peptide was reconstituted in Milli-Q (Millipore, Billerica, MA, USA) water to 2 mg ml^{-1} and was added in 40% excess by mass. This was calculated by multiplying mg of antibody by 0.4. The reconstituted peptide was combined with diluted antibody and incubated for 45 min at 37°C prior to use.

Western blot

Protein was extracted from whole *An. gambiae* 4th-instar larvae using TriZol® (Molecular Research Center, Inc.) according to manufacturer's instructions. Protein samples were prepared and run on a gel according to manufacturer's instructions (NuPage 4–12% Bis-Tris gel, 1.0 mm×12 well; Invitrogen). The protein was transferred to nitrocellulose in transfer buffer (849 μl Milli-Q water, 50 ml 20× NuPAGE transfer buffer, 1 ml NuPAGE antioxidant and 100 ml methanol) at 24 V for 4 h at 4°C. The nitrocellulose was stained with Fast Green (Sigma-Aldrich Corp., St Louis, MO, USA) (40 ml acetic acid, 100 ml methanol, 0.24 g Fast Green, 100 ml water) for approximately 15 min, destained (5:1:5 methanol:HOAc:H_2O) for 15 min and rinsed with deionized water. The individual lanes were separated and blocked with blotto (400 ml TBS, 10 g dry milk, 800 μl Tween-20) for 1 h at room temperature. The primary antibody 5563 was added at a 1:1000 dilution in blotto and rocked at 37°C for 1 h. The blot was washed three times in TBS at room temperature for 5 min each. The secondary antibody [alkaline phosphatase (AP)-conjugated donkey anti-chicken; Jackson ImmunoResearch; West Grove, PA, USA] was added at a 1:250 dilution in blotto and incubated at 37°C for 1 h. The blot was again washed with TBS at room temperature for 3×5 min. The labeled bands were detected using an AP-conjugate substrate kit (Bio-Rad, Hercules, CA, USA) according to manufacturer's instructions.

Immunolocalization

Early 4th-instar mosquito larvae were dissected to separate the gut (including GC, AMG, PMG, MT, ileum and rectum) from the rest of the larvae. The dissected tissue was fixed in a 1:1 solution of hemolymph substitute solution (Clark et al., 1999) and 4% paraformaldehyde in 0.1 mol l^{-1} sodium cacodylate buffer overnight at 4°C. The tissue was then washed twice with TBS for 30 min each at room temperature and incubated in pre-incubation buffer (pre-inc; 1% BSA, 1% normal goat serum, 0.1% Triton X-100 in TBS) for 1–2 h at room temperature. The tissue was incubated with primary chicken antibodies 5340 or 5563 diluted 1:1000 and Na^+/K^+-ATPase mouse monoclonal antibody hybridoma supernatant diluted 1:10 in pre-inc at 4°C overnight. The following day, the tissue was washed approximately 15 times in pre-inc for 30 min each at room temperature. Secondary antibodies (FITC-conjugated donkey anti-chicken and TRITC-conjugated goat anti-mouse; Jackson ImmunoResearch) were diluted 1:250 in pre-inc and incubated with the tissue overnight at 4°C. The tissue was then rinsed twice with pre-inc at room temperature for 30 min each and once with TBS for 30 min at room temperature. To detect muscle, TRITC-conjugated phalloidin (Sigma-Aldrich Corp) was added to the tissue, diluted 1:250 in pre-inc and incubated for 30 min at room temperature. To detect nuclei, the tissue was incubated in the nuclear stain DRAQ-5 (Biostatus, Shepshed, Leicestershire, UK), diluted 1:1000 in pre-inc and incubated for 30 min at room temperature. The tissue was then washed twice with TBS for 30 min each at room temperature and mounted in 60% glycerol in TBS with added phenylenediamine (Sigma-Aldrich Corp.) to diminish signal fading. Signal was visualized using laser scanning confocal microscopy.

Phylogenetic analysis

CA protein alignments were created using ClustalX (http://bips.u-strasbg.fr/fr/Documentation/ClustalX/). Predicted sequences were obtained from the ensembl database (February 2007 release). The alignments were created using ClustalX and trimmed and visualized using GeneDoc (http://www.nrbsc.org/gfx/genedoc/index.html). The phylogeny was prepared using MrBayes (http://mrbayes.csit.fsu.edu/) with the JTT amino acid substitution model and 1.5 million iterations. Trees were visualized using TreeView (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). All nodes are supported with >0.95 posterior probability.

Results

Uniform CA naming system

Table 1 lists the ensembl protein (ENSANGP), transcript (ENSANGT) and gene (ENSANGG) numbers, based on the February 2007 release, as well as the new naming convention that we propose. In this new convention, each name begins with a species identifier, Ag, followed by a two-letter protein abbreviation, CA, or CA-RP in the case of the CA-related proteins (CA-RPs). ENSANGP numbers were listed in ascending numerical order and α-CAs were named sequentially starting with AgCA1. The β-CA was named AgCAb. Also recorded are the GenBank accession numbers of those CAs that our laboratory has fully cloned and sequence confirmed, including AgCA-RP2, which has not yet been submitted to NCBI.

Cloning of full-length AgCA9 from An. gambiae midgut

An 811 base pair (bp) internal fragment was cloned from the *An. gambiae* larval midgut using primers designed according to the partial cDNA sequence predicted by ensembl. Using gene-specific sequences from this fragment, rapid amplification of cDNA ends (RACE) was used to amplify and sequence the 3′ and 5′ ends. The cDNA spanned both the start and stop codons and was determined to be full length by the presence of a stop codon in the 5′ UTR and the presence of a 3′ poly-A tail. The full-length cDNA was determined to be 831 bp, corresponding to a protein product of 276 amino acids with a molecular mass of 31.5 kDa (as determined by http://bioinformatics.org/sms/). This was submitted to the GenBank database and allocated the accession number DQ518576. The clone belongs to the α-CA family and is located on chromosome 3R

Table 1. *Anopheles gambiae carbonic anhydrase (CA) new convention*

	ENSANGP000000	ENSANGT000000	ENSANGG000000	New convention	Accession number
α-CA	01278	01278	01096	AgCA1	
CA-RP	01574	01574	01335	AgCA-RP2	
α-CA	10017	10017	07528	AgCA3	
α-CA	11013	11013	08524	AgCA4	
CA-RP	11908	11908	09419	AgCA-RP5	
α-CA	12957	12957	10468	AgCA6	DQ518577
α-CA	14919	14919	12430	AgCA7	
α-CA	18999	18999	16510	AgCA8	
α-CA	19212	19212	16723	AgCA9	DQ518576
α-CA	21313	21313	18824	AgCA10	AY280612
α-CA	21739	21739	19250	AgCA11	AY280613
β-CA	29115	28335	15071	AgCAb	EF065522

This table lists the ensembl (www.ensembl.org) protein (ENSANGP; column 2), transcript (ENSANGT; column 3), and gene (ENSANGG; column 4) numbers associated with the 11 predicted α-CAs, including the two predicted CA-related proteins (CA-RPs), and the single β-CA encoded in the genome. The protein numbers were listed in ascending numerical order, and new CA conventions were assigned sequentially. Also noted are the GenBank accession numbers of those CAs our laboratory has fully cloned and sequence confirmed, including AgCA-RP2, which has not yet been submitted to NCBI. The ensembl numbers are based on the February 2007 release.

(www.vectorbase.org). A protein alignment between AgCA9 and several other *An. gambiae* α-CAs cloned by the Linser laboratory as well as *H. sapiens* CAII and *D. melanogaster* CAH1 demonstrated that AgCA9 has significant homology at the α-CA active site (Fig. 1). Specifically, note the conserved histidine residues essential for α-CA activity: His-94, His-96 and His-119 (numbering is relative to HCAII).

Detection of RNA expression in alimentary canal regions

Quantitative PCR (qPCR) and *in situ* hybridization were used to detect mRNA within the mosquito larval alimentary canal. AgCA9 mRNA expression was evaluated using qPCR from cDNA derived from whole larval alimentary canal, GC, AMG, PMG, MT and rectum. The results were normalized to the *An. gambiae* 18s ribosomal RNA gene and reported relative to

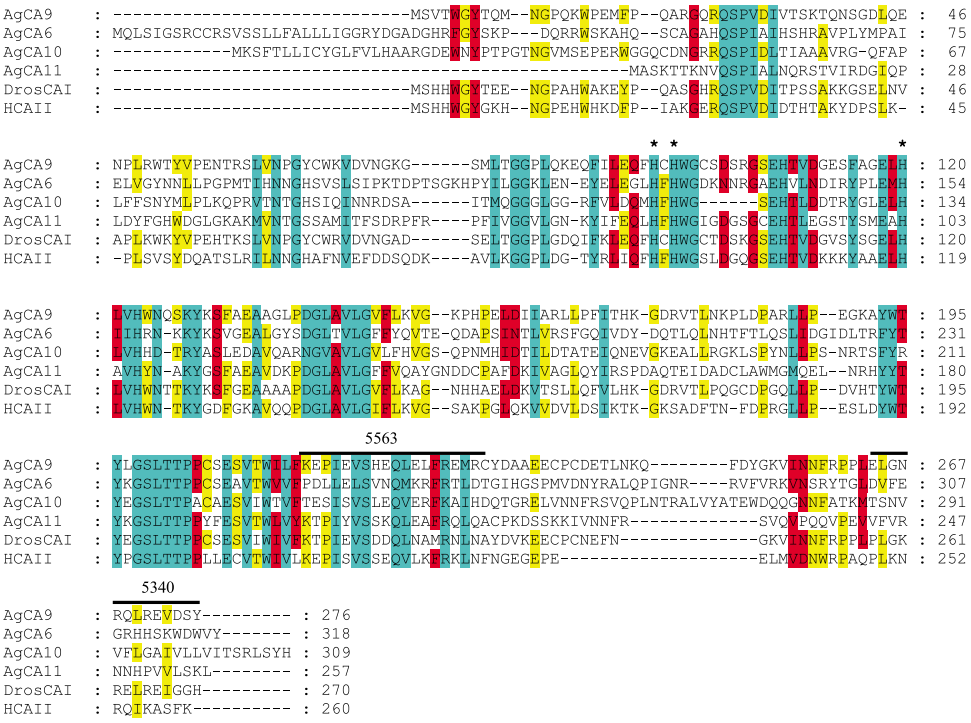

Fig. 1. Alignment of AgCA9 with AgCA6, AgCA10 and AgCA11, formally cloned by the Linser laboratory. Also included are *Homo sapiens* CAII (HCAII) and *Drosophila melanogaster* CAI (DrosCAI). Alignment was performed in ClustalX and visualized using Genedoc. Similarity groups were enabled using the default scoring matrix, Blosum 62 matrix. Regions with high similarity are highlighted in blue (100%). Regions of lesser similarity are highlighted in red (>80%) and yellow (>60%). The three histidines essential for α-CA activity are indicated with a star (HCAII his-94, his-96, his-119). The black bars indicate antigenic sites against which antibodies (5340 and 5563) were generated.

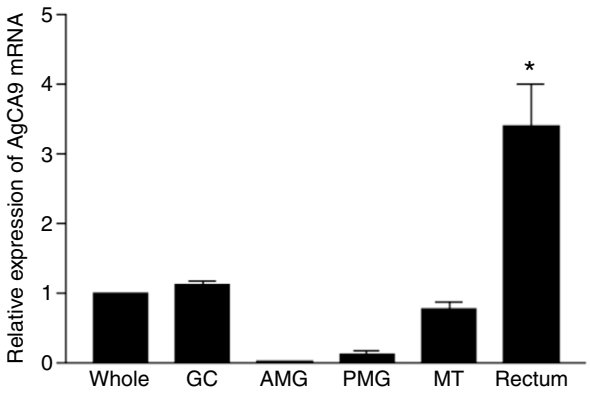

Fig. 2. qPCR was used to measure expression of AgCA9 mRNA in alimentary canal regions – gastric caeca (GC), anterior midgut (AMG), posterior midgut (PMG), Malpighian tubules (MT) and rectum – relative to whole alimentary canal. All values were normalized to an 18s RNA endogenous control. 'Whole' was artificially set to a value of 1.0 in this graph, with all other samples expressed as a proportion of 'whole'. Results indicate that AgCA9 mRNA is significantly more abundant in the rectum samples (*P<0.001 as determined by one-way analysis of variance). Error bars indicate standard deviation between three individual qPCR runs.

whole larval alimentary canal (Fig. 2). qPCR detected RNA expression in every region tested; however, the rectum was the region that expressed AgCA9 significantly above whole larval alimentary canal levels.

In situ hybridization was performed using full-length DIG-labeled RNA probes to detect AgCA9 RNA in the early 4th-instar whole-mount *An. gambiae* larval guts and carcass (including all remaining tissues of the mosquito after the alimentary canal is removed) (Fig. 3). Full-length probes were shown to generate a more specific representation of mRNA expression than cleaved probes (E. Meleshkavich, personal communication). The most intense AgCA9-specific staining of the alimentary canal was seen throughout the cytoplasm of the cells of the GC, TR and rectum, with weaker staining seen in the cells of the PMG. Currently, there is no method of morphologically discriminating the TR in the dissection of alimentary canal regions without electron microscopy. Therefore, RNA from this region is diluted into AMG and PMG samples and cannot be distinguished using qPCR. The most abundant staining of the carcass was seen in the fat body and muscle. No staining was seen in the alimentary canal or carcass using the sense probe.

Verification of antibody specificity

Two antibodies were generated by Aves Labs, Inc. (Tigard, OR, USA) for use in western blotting and immunohistochemistry. The first antibody (from hen 5340), was generated against the BSA-conjugated peptide CZELGNRQLREVDSY. The second antibody (from hen 5563) was generated against the BSA-conjugated peptide KEPIEVSHEQLELFREMRC and affinity purified. Fig. 1 illustrates the antigenic sites on the AgCA9 protein against which these antibodies were generated. Both specifically recognized AgCA9 on a western blot (e.g. Fig. 4A); however, the antibody from hen

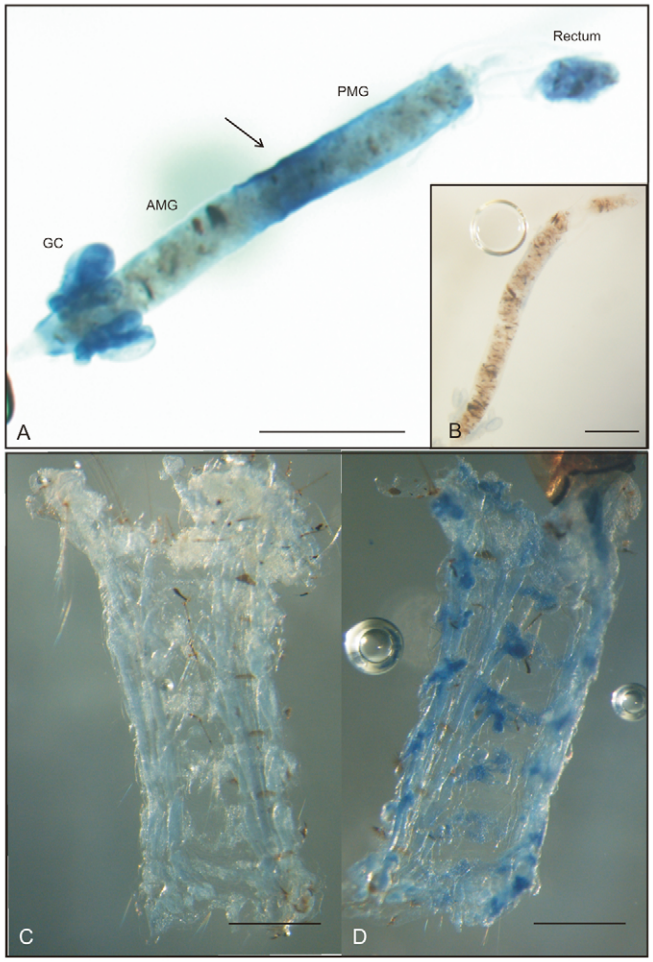

Fig. 3. *In situ* hybridization was used to detect mRNA in whole larval alimentary canals and carcass. A full-length DIG-labeled RNA probe was used to detect AgCA9 mRNA in whole-mount larvae. Antisense probe generated intense staining in the GC, the transitional region between the anterior and posterior midguts (indicated by arrow) and the rectum of the larval alimentary canal, with weaker staining seen in the cells of the PMG (A). Carcass showed the most intense staining in the muscle fibers and fat body (D). Sense probe did not significantly stain any areas of the alimentary canal (B) or carcass (C). For abbreviations, see Fig. 2 legend. Scale bars: 400 μm.

5340 recognized a second protein as well. Only results that were consistent between the two antibodies are reported here. Western blot results for antibody 5563 are shown in Fig. 4A. The blot was performed using protein extracted from whole *An. gambiae* 4th-instar larvae. The antibody detected a single specific band at 31.5 kDa representing AgCA9. The identity of the band was then verified by blocking the antibody with reconstituted peptide. When blocked, the antibody failed to detect any bands, further confirming that the antibody recognized AgCA9 in a specific manner. Specificity of protein localization seen in longitudinal sections (as discussed in the next section) was also verified using a preadsorption blocking assay. When the antibody was blocked with reconstituted peptide, it failed to detect AgCA9 protein (Fig. 4B–E). Apparent expression in the exoskeleton is nonspecific (Fig. 4B,C).

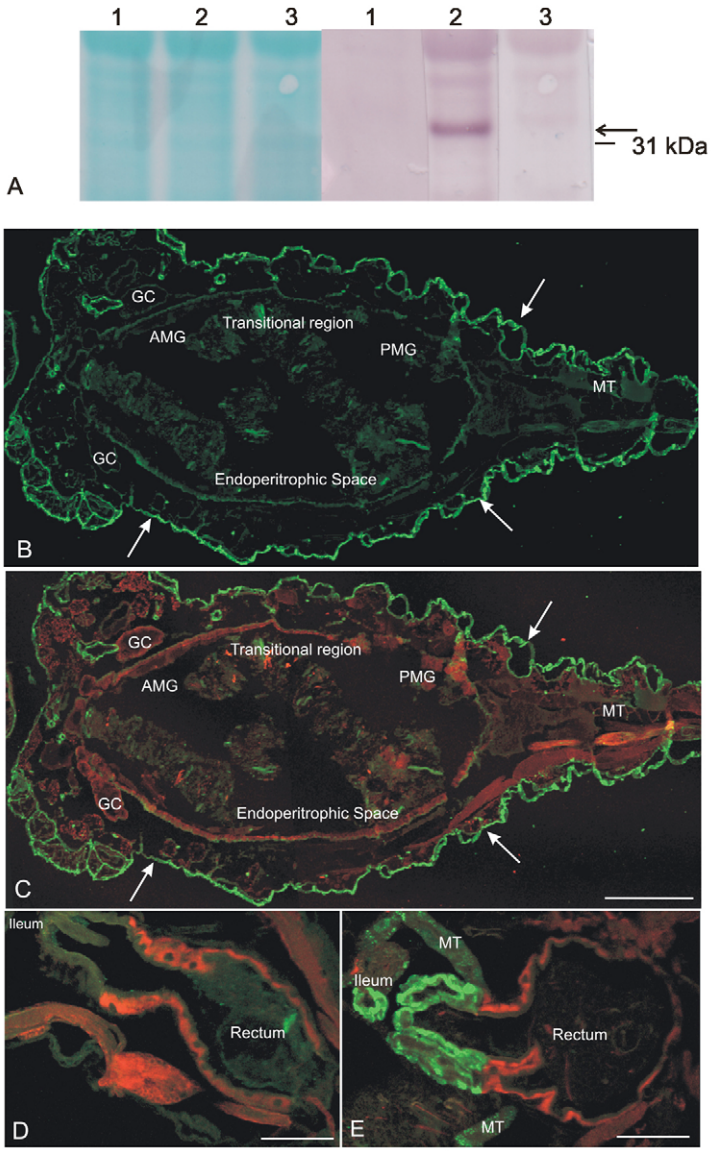

Fig. 4. The specificity of the affinity-purified antibody 5563 was verified using a western blot (A) and immunohistochemistry (B–E). Part A shows the results of an antibody adsorption assay. The left panel of the image is the Fast Green staining of total protein to ensure equal loading. Antibody was pre-incubated with peptide prior to detection of protein. The western blot illustrates the detection of protein with pre-immune IgY (lane 1), affinity-purified antibody 5563 IgY (lane 2) and peptide-blocked IgY (lane 3). Images B–E illustrate immunohistochemistry results obtained using affinity-purified antibody 5563 IgY (E) and peptide-blocked IgY (B,C,D) in longitudinal sections of whole larvae embedded in paraffin. The images were generated on a laser confocal microscope (Leica SP2). B and C are images of the same section of larval alimentary canal (without the rectum) with two different fluorochromes (FITC = green = AgCA9; TRITC = red = Na^+/K^+-ATPase). D and E are images of the rectum positioned such that the anterior end is to the left. Na^+/K^+-ATPase immunostaining was used as a counter-stain to better visualize the regions of the alimentary canal in images C–E. The pre-adsorbed antibody (B–D) failed to detect AgCA9 protein compared with antibody not treated with peptide (E; also Fig. 6A). Apparent exoskeleton staining is non-specific (B,C; arrows). For abbreviations, see Fig. 2 legend. Scale bars, 150 μm (B,C); 75 μm (D,E).

Detection of AgCA9 protein in An. gambiae larvae

Once the specificity of the affinity-purified antibody 5563 was verified, a western blot was used to determine the presence of AgCA9 protein in the individual regions of the alimentary canal including GC, AMG, PMG, MT and rectum (Fig. 5). AgCA9 protein was most abundant in the GC and rectum, followed by the MT. AgCA9 detection in the AMG and PMG was relatively weak.

Both whole-mount larval preparations and longitudinal sections of whole larvae embedded in paraffin were used to detect AgCA9 protein with antibodies 5563 and 5340. Specific protein was detected in the ectoperitrophic fluid and in the cells of the TR, MT and rectum, as will be discussed in more detail below (Fig. 6A).

Ectoperitrophic fluid

While detection of AgCA9 protein on a western blot indicated significant protein levels in the GC, immunohistochemical results within the cells of the GC were variable. In most preparations, AgCA9 was detected in the proteinaceous matrix that occupied the lumen of the GC and lined the alimentary canal (from the GC through the PMG) within the ectoperitrophic space but was not detected in the food bolus (Fig. 6A). AgCA9 protein is indeed within the ectoperitrophic space and not in the peritrophic matrix (PM). The proteinaceous matrix expressing AgCA9 was measured to be 10–27 μm thick in both longitudinal and cross-sections of whole larvae embedded in paraffin (e.g. Fig. 6A), making it 10–30 times thicker than the PM in *Ae. aegypti* (Clements, 1992). This proteinaceous matrix is clearly non-cellular and most likely comprises the ectoperitrophic fluid, which is known to have an important role in insect digestion (Terra and Ferreira, 1981; Terra et al., 1979). Both vitally and as a result of fixation, there are areas along the midgut where the PM lies very close to the epithelial cells. This results in a very thin ectoperitrophic space where the ectoperitrophic fluid is immediately adjacent to the apical membrane of the epithelial cells. Based on numerous immunolocalization experiments, we do not believe that AgCA9 protein is located on the apical membrane of any midgut epithelial cells but is confined to the ectoperitrophic fluid.

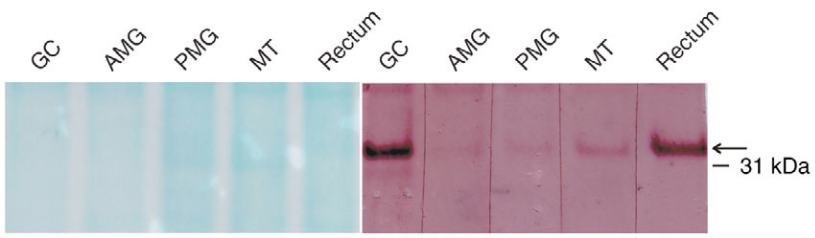

Fig. 5. A Western blot was used to determine AgCA9 protein localization in larval alimentary canal regions including GC, AMG, PMG, MT and rectum. The left panel of the figure is the Fast Green staining of total protein to ensure equal loading. The strongest AgCA9 signal was seen in both the GC and rectum. CA protein expression was less abundant in the MT and minimal in the AMG and PMG. AgCA9 is indicated by an arrow. For abbreviations, see Fig. 2 legend.

Transitional region

The cells of the TR clearly express AgCA9 mRNA as demonstrated by *in situ* hybridization; however, protein detection was variable. In many preparations, AgCA9 protein was observed in association with the periphery of the nucleus (Fig. 6B). Na^+/K^+-ATPase was found by others to have regionally specific membrane localization in *Ae. Aegypti*, with a switch in polarity from the apical membrane in the AMG to the basal membrane in the PMG (Patrick et al., 2006). This polarity switch is also evident in *An. gambiae* (B. A. Okech, personal communication) and can be used to identify the AMG and PMG regions. The beginning of the TR is marked by the switch in localization of Na^+/K^+-ATPase to the basal membrane (Fig. 6C, arrowhead). The AgCA9 protein was restricted to the nuclei of the cells of the TR and did not extend into the AMG. No marker for the end of the TR and beginning of the PMG is known at this time. Counterstaining with the nuclear stain DRAQ-5 confirmed that CA protein was confined to the periphery of the nucleus but was absent from the center region of the structure (Fig. 6D).

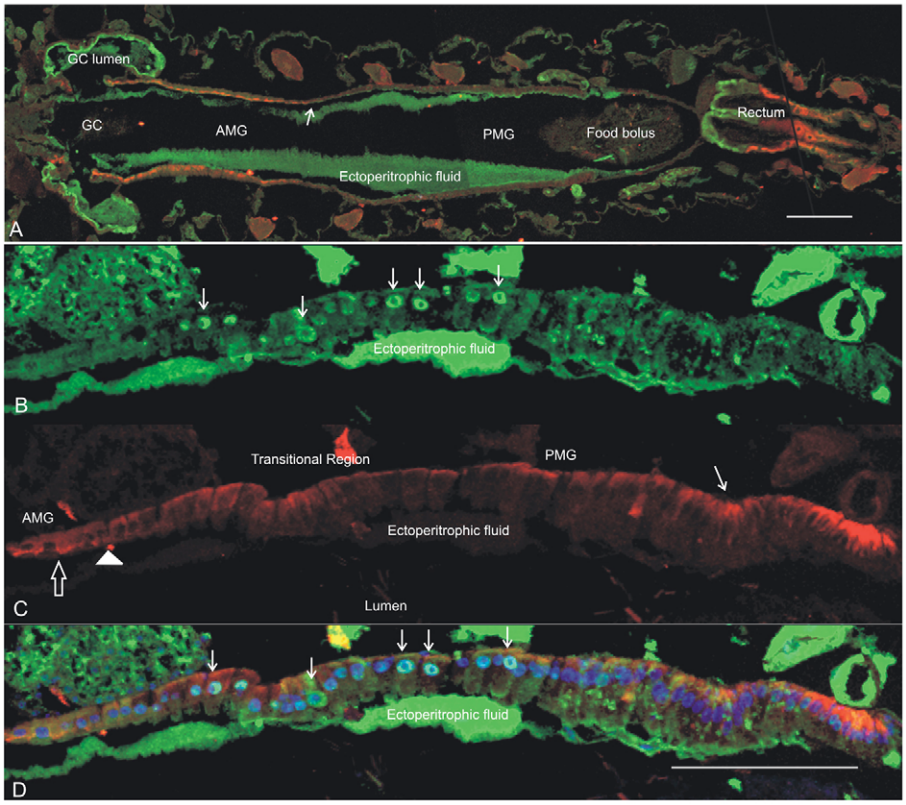

Fig. 6. Immunohistochemistry was performed to detect AgCA9 and Na^+/K^+-ATPase protein in longitudinal sections of whole larvae embedded in paraffin. The images were generated on a laser confocal microscope (Leica SP2). The anterior end of the larvae is towards the left. B–D are images of the same section with the three different fluorochromes (FITC = green = AgCA9; TRITC = red = Na^+/K^+-ATPase; Cy5 = blue = DRAQ-5 nuclear stain, respectively). AgCA9 protein detection was variable in the epithelial cells of the GC but was consistently seen in the proteinaceous matrix that fills the GC lumen and lines the alimentary canal within the ectoperitrophic space (ectoperitrophic fluid) (A). Na^+/K^+-ATPase was used as a counter-stain to identify the regions of the alimentary canal (A,C,D). Na^+/K^+-ATPase is localized to the apical membrane in the cells of the AMG (C, open arrow) and to the basal membrane in the cells of the PMG (C, arrow). In the vicinity of the switch from apical to basal localization is the beginning of the TR (A, arrow; C, arrowhead). AgCA9 detection in the transitional region reveals an association with the periphery of the nucleus (B,D; e.g. arrows). Nuclear localization was confirmed by counterstaining with the nuclear stain DRAQ-5 (D, blue). For abbreviations, see Fig. 2 legend. Scale bars: 150 μm (A); 172.2 μm (B–D).

Malpighian tubules

AgCA9 protein was detected in a punctate pattern within the principal cells of the MT, with stellate cells appearing devoid of protein (Fig. 7). The protein appeared to associate with cytoplasmic inclusions throughout the MT and did not localize to either the membrane or nucleus.

Rectum

A patch of epithelial cells covering roughly one quarter of the rectum and positioned on the dorsal side of the apical extreme of this alimentary canal region showed very intense and specific AgCA9 staining. These cells will henceforth be referred to as ‘dorsal anterior rectal cells’, or DAR cells. This localization pattern was recognized first by the antibody 5340 in whole-mount larval alimentary canal preparations (Fig. 8A), and specificity to AgCA9 was verified using affinity-purified antibody 5563 in paraffin sections (Fig. 8B). To compare and contrast this staining pattern, a monoclonal antibody to Na^+/K^+-ATPase was used, which was found by others

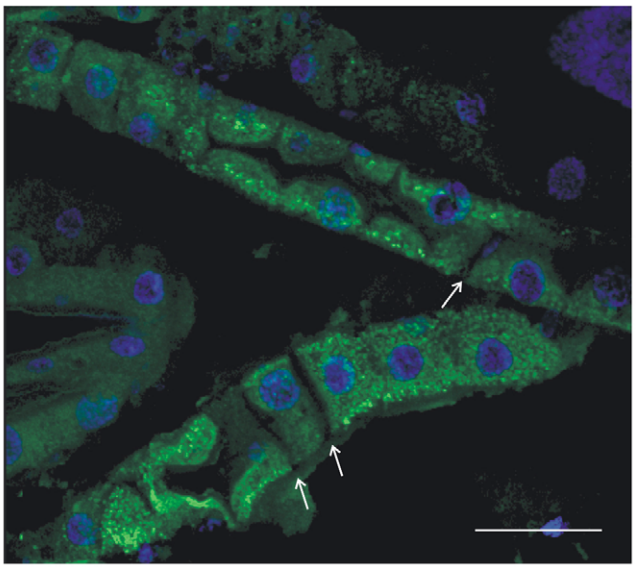

Fig. 7. Immunohistochemistry of AgCA9 in Malpighian tubule (MT) sections. The confocal image demonstrates punctate AgCA9 protein localization within the principal cells of the MT (green). Stellate cells (e.g. arrows) do not appear to contain AgCA9 protein. The nuclei were detected using nuclear stain DRAQ-5 (blue). Scale bar: 47.62 μm.

working in parallel to produce a negative staining pattern in this region relative to the AgCA9 in whole-mount larval preparations (B. A. Okech, personal communication). Indeed, this basal membrane marker protein produced a reversed image of the AgCA9 pattern (Fig. 8C). Localization in paraffin sections of the rectum showed AgCA9 protein to be clearly cytoplasmic whereas the Na^+/K^+-ATPase localized to the very extensive basal infoldings as described by Patrick et al. (Patrick et al., 2006). The unusual banding pattern seen in the DAR cells was investigated by counterstaining with phalloidin to reveal circumferential muscles surrounding the rectum (Fig. 8D).

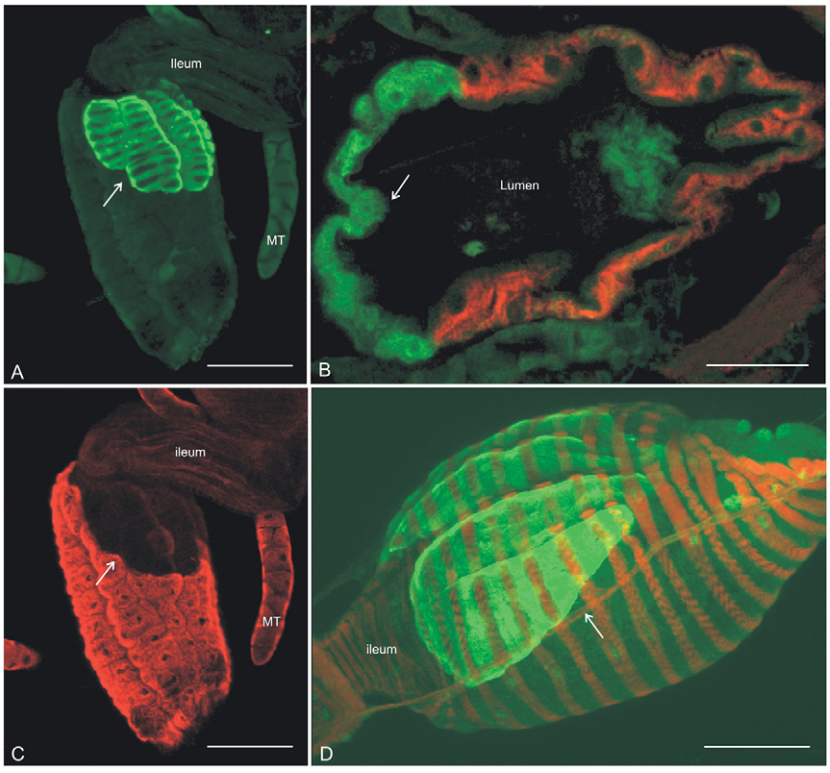

Fig. 8. Immunohistochemistry of AgCA9 protein in whole-mount larvae (A,C,D) and longitudinal paraffin sections of larvae (B). The figure shows laser confocal microscope images of *An. gambiae* larval rectum immuno-stained with chicken antibodies 5340 (green; A,D) and 5563 (green; B) and mouse monoclonal antibodies against Na^+/K^+-ATPase (red; B,C). The ileum is indicated at the anterior end of the rectum (A,C,D). AgCA9 protein localized to the DAR cells exclusively (A,B,D; arrow). The banding pattern seen in the DAR cells of whole mounts of the rectum is due to the circumferential muscles of the rectum. This is better seen in D, which shows muscle stained with phalloidin (red). Na^+/K^+-ATPase localized to the entire rectum, with the exception of the DAR cells (B,C). DAR cells are indicated with an arrow in each image. Scale bars: 150 μm (A,C); 75 μm (B,D).

Phylogenetic analysis

A phylogenetic tree comparing predicted and cloned CA transcripts from *H. sapiens*, *D. melanogaster*, *Ae. aegypti* and *An. gambiae* showed a general segregation of human and insect CAs (Fig. 9). This separation was not seen in the case of the CA-RPs, proteins that have lost an essential histidine residue at the active site and are no longer active. Interestingly, AgCA9 was the sole *An. gambiae* CA that clustered with *H. sapiens* CAs, along with its *D. melanogaster* and *Ae. aegypti* homologues. Within the *H. sapiens* CAs, clustering occurred between proteins with the same or similar subcellular localizations. There was a distinct separation between the secreted, membrane-bound and transmembrane CAs. Cytosolic and mitochondrial CAs formed a fourth cluster.

Discussion

The ensembl database predicts a total of 11 α-CA genes and one β-CA gene to be present in the *An. gambiae* genome, six of which have already been fully cloned by the Linser laboratory (e.g. Table 1) (Linser et al., 2003) (T. J. Seron, personal communication; K.E.S., unpublished data). The cloning and characterization of each of the remaining six CAs is necessary to determine the role this family plays in pH regulation. We were driven to investigate these CAs by our hypothesis of an extracellular CA in the lumen of the alimentary canal. Here, we report the cloning and characterization of a novel cytoplasmic-like CA, AgCA9, from the alimentary canal of the larval malaria mosquito *An. gambiae*. The full-length transcript of this gene is 831 bps; the resulting protein is 276 amino acids, generating a 31.5 kDa protein. The clone belongs to the α-carbonic anhydrase family and is located on chromosome 3R (www.vectorbase.org).

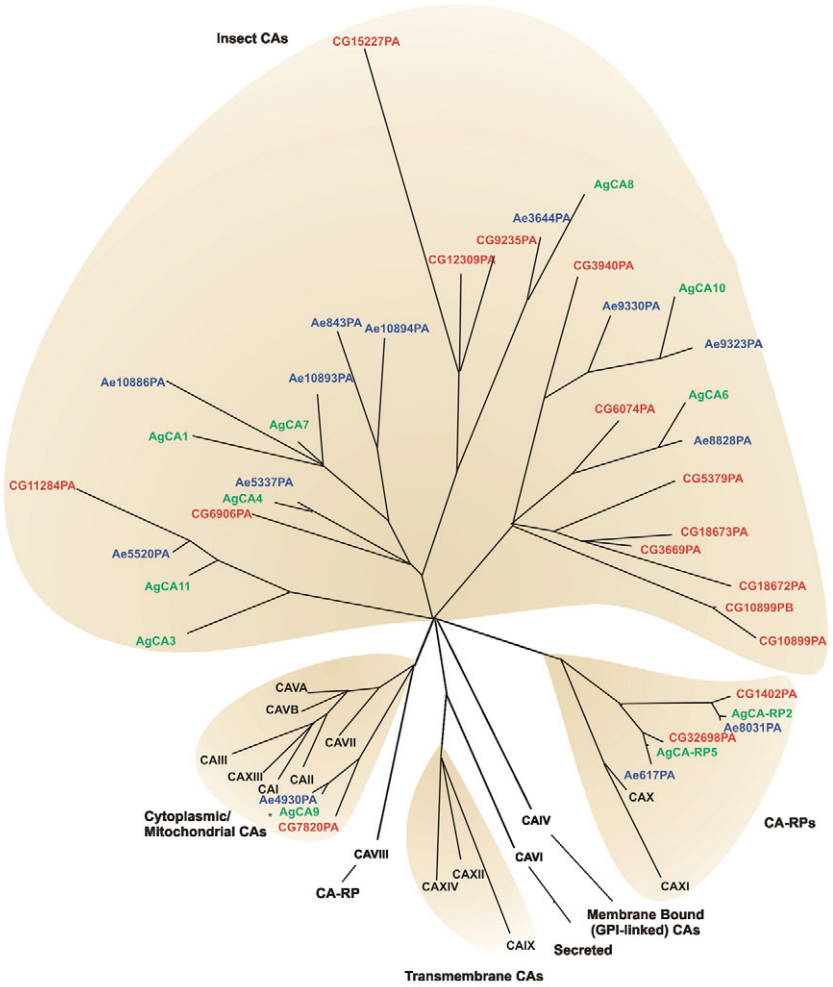

Fig. 9. A phylogenetic analysis was generated for members of the CA family in *Homo sapiens* (black), *Drosophila melanogaster* (red), *Aedes aegypti* (blue) and *Anopheles gambiae* (green) genomes. Alignments were created using ClustalW and trimmed and visualized using Genedoc. Phylogeny was prepared using MrBayes, with the JTT amino acid substitution model and 1.5 million iterations. Trees were visualized using Treeview. The tree suggests that amplification of insect CAs most likely took place after the divergence of deuterostomes and protostomes as the insect CAs overwhelmingly clustered together. CA-RPs are conserved between protostomes and deuterostomes and are likely to be ancient proteins. Interestingly, AgCA9 (asterisk) and its *Drosophila* and *Aedes* homologues were the sole insect CA that clusters with human CAs, indicating that it most closely resembles the primal CA from which other CAs branched.

Together, qPCR and *in situ* hybridization revealed that AgCA9 mRNA is most abundant in the GC, the TR and the rectum. These data were supported by data generated from microarrays performed using mRNA from the various regions of the *An. gambiae* alimentary canal [GC, AMG, PMG, hindgut (HG)] (Neira et al., in press). Separate data were not available for MT and rectum but were combined into one sample labeled 'HG'. The microarray compared the expression levels of approximately 14 000 *An. gambiae* genes (Affymetrix GeneChip® *Plasmodium*/*Anopheles* Genome Array; Affymetrix Inc., Santa Clara, CA, USA) in each region of the alimentary canal as compared with whole insect. The microarray detected a higher expression of AgCA9 in the GC and HG than that of whole insect (Neira et al., 2006).

To test the theory of an extracellular CA, antibody probes against AgCA9 were used to determine protein distribution within the alimentary canal. AgCA9 was detected in the proteinaceous matrix that occupies the GC lumina as well as the length of the midgut in the ectoperitrophic space. Additionally, AgCA9 protein was found in the cells of the TR and MT and in the cytoplasm of the DAR cells within the rectum.

There are obvious discrepancies between the determination of AgCA9 mRNA and protein localization. These can be explained simply by recalling that protein localization does not necessarily mirror mRNA transcription. For example, MT were found to have very little AgCA9 mRNA; however, we consistently saw protein in MTs of paraffin sections. A developmental shift in mRNA and protein production could account for these differences. All experiments were performed on early 4th-instar larvae. It is possible that AgCA9 mRNA is expressed in the MT of earlier larval stages and then wanes as the larvae near pupation; if AgCA9 has a slow protein turnover rate, this would lead to the presence of protein in the absence of mRNA expression.

Ectoperitrophic fluid

According to one view of pH regulation in the mosquito alimentary canal, CA catalyzes the production of bicarbonate in the cells of the midgut, which is then translocated into the lumen by a plasma membrane anion exchanger. Our failure to locate this anion exchanger led us to hypothesize the existence of an extracellular CA in the lumen of the alimentary canal. Indeed, immunohistochemistry revealed AgCA9 protein in the proteinaceous matrix (ectoperitrophic fluid) of the ectoperitrophic space within the GC lumina and down the length of the alimentary canal, including the AMG, TR and PMG.

AgCA9 protein in the GC and along the length of the midgut may serve as a source of bicarbonate and carbonate ions with a maximum buffer capacity at both pHs 8.2 and 10.5, respectively. Mosquito alimentary canal epithelial cells are metabolically active, producing carbon dioxide (CO_2). CO_2 can diffuse freely across the cellular membrane and into the ectoperitrophic space, where CA can catalyze its conversion to bicarbonate. Bicarbonate can buffer the lumina of the GC and PMG to a pH around 8.0. Moreover, in the AMG epithelium, a basally located V-ATPase is positioned to move H^+ ions vectorally out of the cells (Zhuang et al., 1999), resulting in a transepithelial potential that is lumen-negative (Clark et al., 1999). This likely results from the combined activities of the V-ATPase and a $K^+/2H^+$ antiporter

(Lepier et al., 1995), which produce a net movement of protons into the hemolymph in the AMG. This loss of luminal H^+ should result in the deprotonation of bicarbonate, forming carbonate. This, combined with a strong cation such as a potassium or sodium ion, would then provide the basis for buffering at a pH near 10.5. Thus, the control of luminal midgut pH is not specifically based on the physical disposition of CA but on the proton gradient generated within the lumen. In caterpillars, the ectoperitrophic space was found to be equal in pH to the endoperitrophic space (Gringorten et al., 1993). Thus, the same pH gradient exists in both compartments of the lumen, and bicarbonate/carbonate in the ectoperitrophic space could lead to a buffering of the entire lumen.

How does a cytoplasmic-like protein, one that is not predicted to have a signal sequence, end up in the extracellular ectoperitrophic fluid? We suggest AgCA9 protein is produced in the cells of the GC (recall that the GC was found to be enriched in AgCA9 mRNA) and secreted into the ectoperitrophic space by a type of exocytosis. There are several methods of secretion that do not require a signal sequence that are often utilized to export digestive enzymes in the cells of insect midguts. Merocrine secretion is a classic form of exocytosis, occurring when membrane-bound vesicles containing soluble proteins open onto the surface of the cell, allowing the proteins to be secreted into the lumen (Hung et al., 2000). Alternatively, apocrine release occurs when a portion of the plasma membrane buds off the cell, containing the proteins (*ibid*).

Notably, there is an unexpected lack of accumulation of AgCA9 protein within the food bolus. The peritrophic matrix of *An. gambiae* larvae is permeable to 148 kDa particles (Edwards and Jacobs-Lorena, 2000) and would be expected to allow 31.5 kDa AgCA9 to freely diffuse into the food bolus. However, the native enzyme conformation of AgCA9 is unknown at this time and could be oligomeric. Additionally, the enzyme may be complexed with other enzymes, making it larger, or may associate with the peritrophic matrix and accumulate at this junction without passing through to the food bolus.

Transitional region

AgCA9 mRNA clearly localized to the TR using *in situ* hybridization; however, the protein localization was not as clear, appearing to localize to the periphery of the nuclei of the cells in this area. The unusual localization of AgCA9 to the nuclei is not unprecedented. There are many papers that have noted CA in the nucleus using both immuno- and enzyme-histochemical methods (Hansson, 1967; Lutjen-Drecoll and Lonnerholm, 1981; Anderson et al., 1982; Brown et al., 1983; Brown and Kumpulainen, 1985; Toyosawa et al., 1996). These papers make no claims as to the validity of these findings and most relate enzyme histochemical detection to the fact that the nuclei act as crystallization centers for the reaction products of Hansson's cobalt precipitation method (Ridderstråle, 1991). We observed AgCA9 nuclear localization using immunohistochemical analyses that are not based on enzymatic activity, and hence nuclear CA remains a physiological mystery.

The TR has recently been described as a unique region in the alimentary canal that was previously thought to be part of the PMG. This region is described as having a continuous morphological change between the AMG and PMG in contrast to a mixture of cells from each region (Clark et al., 2005). The cells of this region have long microvilli and a high density of mitochondria (Clark et al., 2005), suggesting a transport role involving a high rate of ATPase activity in *Ae. aegypti*. A distinct band of GPI-linked CA mRNA has previously been described in the anterior end of the PMG as well as an increased mRNA expression of an anion exchanger, AgAE1, in this region (Seron et al., 2004) (T. J. Seron, personal communication). This localization to the nuclei of the TR identifies a differential quality of these cells compared with the rest of the alimentary canal. The beginning of the TR demarcates the area at which the highly alkaline pH of the AMG drops to near-neutral values within the lumen of the alimentary canal. We predict that these cells have an important role in the de-alkalization of the alimentary canal. At this time, no explanation is evident for the association of AgCA9 with the nuclei of these cells.

Malpighian tubules

MTs are important components of the mosquito osmoregulatory system, maintaining hemolymph volume and composition (Bradley, 1987). *An. gambiae* larvae are equipped with five MT, which are composed of two cell types: principal cells and stellate cells. The larger principal cells contained AgCA9 protein associated with membranes of vesicular cytoplasmic inclusions, while no such protein was detected in the smaller stellate cells. Palatroni et al. localized CA activity to vesicular cytoplasmic inclusions within the MT of *Culex pipiens* using Hansson's method for histochemical localization (Palatroni et al., 1981). They noted that CA activity was only evident at the level of the membranes of these inclusions and was lacking on the cellular membrane, nucleus and other cytoplasmic structures. The cytoplasm of principal cells is densely packed with membrane-limited vesicles containing concretion bodies – metallo-organic aggregates of Ca^{2+}, Mg^{2+} and K^+ that have roles in metal ion storage as well as transepithelial transport. Stellate cells notably lack these concretion bodies (Beyenbach, 2003; Clements, 1992). AgCA9 protein detection mirrors the enzymatic pattern shown by Palatroni et al. (Palatroni et al., 1981) and possibly represents association with concretion bodies.

DAR cells

Meredith and Phillips analyzed the ultrastructure of freshwater *vs* saltwater mosquito larvae, using *Ae. aegypti* and *Aedes campestris*, respectively, as representative mosquitoes. They specifically stated that, whereas saltwater mosquito larvae possess a rectum that is divided into two regions, the rectum of freshwater mosquito larvae is uniform in structure and function, similar to the anterior rectum of saltwater breeders (Meredith and Phillips, 1973). This has been supported by light microscopy of two other species of mosquito, *Aedes albopictus* (Asakura, 1970) and *Aedes detritus* (Ramsay, 1950), as well as with hemipteran larvae, *Hydrometra stagnorum* and *Halosalada lateralis* (Goodchild, 1969). Meredith and Phillips hypothesized that the posterior region of the rectum is unique to saltwater breeders and is used for generating hyperosmotic urine as a way of regulating the high salt intake. The anterior portion of the rectum is thought to selectively resorb ions, water and metabolites produced by the MTs (Meredith and Phillips, 1973).

As primarily a freshwater breeder, *An. gambiae* larvae would be expected to have a uniform rectum. However, AgCA9 clearly localized solely to a subset of cells in the anterior region of the *An. gambiae* rectum, suggesting distinctive functions of these cells in the rectum. The protein was not detected in the entire anterior rectum but was localized to cells on the dorsal side (DAR cells). This cellular pattern is reminiscent of insect 'rectal glands' – specialized groups of rectal epithelial cells that have a major role in water absorption (Wigglesworth, 1932). Although rectal glands are thought to be absent from nearly all larvae (*ibid*), this possibility cannot be excluded.

The DAR cells also differentially expressed other pH- and ion-regulatory proteins such as Na^+/K^+-ATPase, V-ATPase (B. A. Okech, personal communication) and an *An. gambiae* cytoplasmic CA (AgCA11; accession number AY280613) (T. J. Seron, personal communication). This suggests a highly regulated system of pH and ion regulation within the rectum. Clearly, the rectum of this particular freshwater breeder is not uniform, as originally thought, but the specific roles of the DAR cells remain to be determined. Most of the work on the mosquito larval rectum has been performed on members of the subfamily Culicinae, with no information available on the rectum of Anophelinae larvae, including *An. gambiae*. The DAR cells may be unique to the larvae of Anopheline mosquitoes.

Our data suggest that the regions of the *An. gambiae* rectum may have similar roles to the anterior and posterior regions of the rectum in saltwater breeders. The rectum is an important site of pH and HCO_3^- regulation in the larvae of *Aedes dorsalis*, a saltwater mosquito capable of inhabiting hypersaline lakes (HCO_3^- and CO_3^{2-} concentrations as high as 1–2.4 mol l^{-1} and pH values exceeding 10) (Strange et al., 1982). Using microperfused rectum preparations, it was reported that the anterior rectum is a site of CO_2 secretion in the form of a Cl^-/HCO_3^- exchange. Additionally, the authors predicted the involvement of a basolateral 1:1 Cl^-/HCO_3^- exchanger and a CA, based on data obtained using inhibitory compounds. The posterior rectum was found to secrete a hyperosmotic fluid containing Na^+, Cl^- and HCO_3^-, as originally suggested by Meredith and Phillips (Meredith and Phillips, 1973; Strange et al., 1984). Our data suggest similar roles for the rectum in *An. gambiae*, with the DAR cells acting as the anterior rectum. The role of the anterior rectum of saltwater species in CO_2 secretion was demonstrated, but a full explanation of the mechanism was not available at the time. Our data complement the work of Strange et al. (Strange et al., 1984) by demonstrating the existence of a CA in the DAR cells. AgCA9 within these cells can catalyze the conversion of CO_2 to HCO_3^-, which can then be transported into the lumen by an as yet unidentified anion exchanger and excreted. Supporting this hypothesis, it is known that mosquito larvae alkalize their rearing media by excreting bicarbonate (Stobbart, 1971) and it has been shown that this alkalization can be blocked using global CA inhibitors (Corena et al., 2002). The remainder of the cells of the rectum express Na^+/K^+-ATPase, a protein capable of transporting one of the three main ions secreted by the posterior rectum of saltwater mosquitoes.

Phylogeny

A phylogenetic analysis was performed to examine the relationship between CAs in the *H. sapiens*, *D. melanogaster*, *Ae. aegypti* and *An. gambiae* genomes. Alignments were made between known and predicted protein sequences as reported by the ensembl February 2007 release, and a tree was generated. A distinct separation was seen between insect and human CAs. However, CA-RPs showed no such distinction and clustered together. This suggests that the CA-RPs are conserved between protostomes and deuterostomes and that active CA amplification took place after their divergence. Although CA-RPs lack CA activity, they are known to be highly conserved proteins, indicating that they serve a critical cellular function (Tashian et al., 2000). Interestingly, AgCA9 and its *Drosophila* and *Aedes* counterparts were the sole insect CAs that cluster with human CAs. This association suggests that AgCA9 is closer to the primal protein from which other CAs branched. In fact, AgCA9 was closely associated with CAVII, the *H. sapiens* CA, which is the most highly conserved of the active CA isozymes and thought to resemble most closely the ancestral state (Hewett-Emmett and Tashian, 1996; Chegwidden and Carter, 2000). This implies that AgCA9 is a highly conserved protein and likely to play a role in similarly conserved functionalities.

Conclusions

The cloning and characterization of the cytosolic-like α-CA, AgCA9, and its extracellular localization to the ectoperitrophic fluid support the hypothesis that bicarbonate production in the lumen is mediated by CO_2 diffusion followed by the action of an extracellular CA. If AgCA9 actively catalyzes the production of bicarbonate within the midgut lumen, it would suggest that it is a key player in AMG alkalization and therefore is key to survival. The protein was also detected in the cells of the TR, MT and DAR, a previously undescribed region of the rectum. We have shown differential localization of the ionoregulatory protein Na^+/K^+-ATPase in addition to AgCA9 in this region. The fact that DAR cells differentially express a number of pH/ion regulatory proteins suggests an overlooked and important role for these cells in larval pH regulation in Anopheline mosquito larvae. A phylogenetic analysis revealed AgCA9 to cluster with *H. sapiens* CAs and to closely associate with CAVII, the most basal *H. sapiens* CA, implying a highly conserved, and thus important, physiological role. Further studies will be performed to investigate the function of AgCA9, specifically focusing on its role in pH- and ion-regulation.

List of abbreviations

AMG	anterior midgut
AP	alkaline phosphatase
BSA	bovine serum albumin
CA	carbonic anhydrase
CA-RP	carbonic anhydrase-related protein
DrosCAI	*Drosophila melanogaster* carbonic anhydrase 1
GC	gastric caeca
HCAII	*Homo sapiens* carbonic anhydrase II
HG	hindgut
IVM	integrative vector management
MT	Malpighian tubules
PMG	posterior midgut
qPCR	quantitative PCR
TR	transitional region

We wish to thank Dmitri Boudko's laboratory for their help in the generation of cDNA collections. This work is supported by NIH NIAID RO1AI 5098-8.

References

Anderson, R. E., Gay, C. V. and Schraer, H. (1982). Carbonic anhydrase localization by light and electron microscopy. *J. Histochem. Cytochem.* **30**, 1135-1145.

Asakura, K. (1970). Studies on the structural basis for ion- and water-transport by the osmoregulatory organs of mosquitoes. I. Fine structure of the rectal epithelium of *Aedes albopictus* larvae. *Sci. Rep. Kanazawa Univ.* **1**, 37-55.

Berenbaum, M. (1980). Adaptive significance of midgut pH in larval Lepidoptera. *Am. Nat.* **115**, 138-146.

Beyenbach, K. W. (2003). Transport mechanisms of diuresis in Malpighian tubules of insects. *J. Exp. Biol.* **206**, 3845-3856.

Bradley, T. J. (1987). Physiology of osmoregulation in mosquitoes. *Annu. Rev. Entomol.* **32**, 439-462.

Brown, D. and Kumpulainen, T. (1985). Immunocytochemical localization of carbonic anhydrase on ultrathin frozen sections with protein A-gold. *Histochemistry* **83**, 153-158.

Brown, D., Kumpulainen, T., Roth, J. and Orci, L. (1983). Immunohistochemical localization of carbonic anhydrase in postnatal and adult rat kidney. *Am. J. Physiol.* **245**, F110-F118.

Chegwidden, R. W. and Carter, N. D. (2000). Introduction to the carbonic anhydrases. In *The Carbonic Anhydrases: New Horizons* (ed. W. R. Chegwidden, N. D. Carter and Y. H. Edwards), pp. 13-28. Basel: Birkhäuser.

Clark, T. M., Koch, A. and Moffet, D. F. (1999). The anterior and posterior 'stomach' regions of larval *Aedes aegypti* midgut: regional specialization of ion transport and stimulation by 5-hydroxytryptamine. *J. Exp. Biol.* **202**, 247-252.

Clark, T. M., Hutchinson, M. J., Huegel, K. L., Moffett, S. B. and Moffett, D. F. (2005). Additional morphological and physiological heterogeneity within the midgut of larval *Aedes aegypti* (Diptera: Culicidae) revealed by histology, electrophysiology, and effects of *Bacillus thuringiensis* endotoxin. *Tissue Cell* **37**, 457-468.

Clements, A. N. (1992). *The Biology of Mosquitoes*, Vol. 1 (ed. A. N. Clements). London: Chapman and Hall.

Corena, M. P., Seron, T. J., Lehman, H. K., Ochrietor, J. D., Kohn, A., Tu, C. and Linser, P. J. (2002). Carbonic anhydrase in the midgut of larval *Aedes aegypti*: cloning, localization and inhibition. *J. Exp. Biol.* **205**, 591-602.

Dadd, R. H. (1975). Alkalinity within the midgut of mosquito larvae with alkaline-active digestive enzymes. *J. Insect Physiol.* **21**, 1847-1853.

Dow, J. A. T. (1984). Extremely high pH in the biological systems: a model for carbonate transport. *Am. J. Physiol.* **246**, R633-R635.

Edwards, M. J. and Jacobs-Lorena, M. (2000). Permeability and disruption of the peritrophic matrix and caecal membrane from *Aedes aegypti* and *Anopheles gambiae* mosquito larvae. *J. Insect Physiol.* **46**, 1313-1320.

Goodchild, A. J. P. (1969). The rectal glands of *Halosalada lateralis* (Fallen) (Hemiptera: Saldidae) and *Hydrometra stagnoum* (L.) (Hemiptera: Hydrometridae). *Proc. R. Entomol. Soc. Lond. A* **44**, 62-70.

Gringorten, J. L., Crawford, D. N. and Harvey, W. R. (1993). High pH in the ectoperitrophic space of the larval lepidopteran midgut. *J. Exp Biol.* **183**, 353-359.

Hansson, H. P. J. (1967). Histochemical demonstration of carbonic anhydrase activity. *Histochemie* **11**, 112-128.

Hewett-Emmett, D. and Tashian, R. E. (1996). Functional diversity, conservation and convergence in the evolution of the alpha-, beta-, and gamma-carbonic anhydrase gene families. *Mol. Phylogenet. Evol.* **5**, 50-77.

Hung, C., Lin, T. and Lee, W. (2000). Morphology and ultrastructure of the alimentary canal of the oriental fruit fly, *Bactrocera dorsalis* (Hendel) (Dipteria: Tephritidae) (2): The structure of the midgut. *Zool. Stud.* **39**, 387-394.

Lebovitz, R. M., Takeyasu, K. and Fambrough, D. M. (1989). Molecular characterization and expression of the (Na[+]+K[+])-ATPase alpha-subunit in *Drosophila melanogaster*. *EMBO J.* **8**, 193-202.

Lepier, A., Azuma, M., Harvey, W. R. and Wieczorek, H. (1995). K^+/H^+ antiport in the tobacco hornworm midgut: the K^+-transporting component of the K^+ pump. *J. Exp. Biol.* **196**, 361-373.

Linser, P. J., Seron, T. J., Hill, J. and Lehman, H. K. (2003). Characterization of novel carbonic anhydrases in larval mosquitoes. *Am. J. Trop. Med. Hyg.* **69**, 335.

Lutjen-Drecoll, E. and Lonnerholm, G. (1981). Carbonic anhydrase distribution in the rabbit eye by light and electron microscopy. *Invest. Ophthalmol. Vis. Sci.* **21**, 782-797.

Matz, M. (2003). Amplification of representative amounts of cDNA pools from microscopic amounts of animal tissue. *Methods Mol. Biol.* **221**, 103-116.

Matz, M., Shagin, D., Bogdanova, E., Britanova, O., Lukyanov, S., Diatchenko, L. and Chenchik, A. (1999). Amplification of cDNA ends based on template-switching effect and step-out PCR. *Nucleic Acids Res.* **27**, 1558-1560.

Meleshkevitch, E. A., Assis-Nascimento, P., Popova, L. B., Miller, M. M., Kohn, A. B., Phung, E. N., Mandal, A., Harvey, W. R. and Boudko, D. Y. (2006). Molecular characterization of the first aromatic nutrient transporter from the sodium neurotransmitter symporter family. *J. Exp. Biol.* **209**, 3183-3198.

Meredith, J. and Phillips, J. E. (1973). Rectal ultrastructure in salt- and freshwater mosquito larvae in relation to physiological state. *Z. Zellforsch. Mikrosk. Anat.* **138**, 1-22.

Neira, M. O., VanEkeris, L., Corena Mc-Leod, M. P. D. and Linser, P. J. (in press). A micro-array based analysis of transcriptional compartmentalization in the alimentary canal of *Anopheles gambiae* (Diptera: Culicidae) larvae. *J. Ins. Mol. Biol.*, in press.

Palatroni, P., Gabrielli, M. G. and Scattolini, B. (1981). Histochemical localization of carbonic anhydrase in Malpighian tubules of *Culex pipiens*. *Experientia* **37**, 409-411.

Patrick, M. L., Aimanova, K., Sanders, H. R. and Gill, S. S. (2006). P-type Na^+/K^+-ATPase and V-type H^+-ATPase expression patterns in the osmoregulatory organs of larval and adult mosquito *Aedes aegypti*. *J. Exp. Biol.* **209**, 4638-4651.

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* **29**, 2002-2007.

Ramsay, J. A. (1950). Osmotic regulation in mosquito larvae. *J. Exp. Biol.* **27**, 145-157.

Ridderstårle, Y. (1991). Localization of carbonic anhydrase by chemical reactions. In *The Carbonic Anhydrases: Cellular Physiology and Molecular Genetics* (ed. S. J. Dodgson, R. E. Tashian, G. Gros and N. D. Carter), pp. 133-144. New York: Plenum Press.

Seron, T. J., Hill, J. and Linser, P. J. (2004). A GPI-linked carbonic anhydrase expressed in the larval mosquito midgut. *J. Exp. Biol.* **207**, 4559-4572.

Stobbart, R. H. (1971). Evidence of Na^+/H^+ and Cl^-/HCO_3^- exchanges during independent sodium and chloride uptake by the larvae of the mosquito *Aedes aegypti*. *J. Exp. Biol.* **54**, 19-27.

Strange, K., Phillips, J. E. and Quamme, G. A. (1982). Active HCO_3^- secretion in the rectal salt gland of a mosquito larva inhabiting $NaHCO_3$-CO_3 lakes. *J. Exp. Biol.* **101**, 171-186.

Strange, K., Phillips, J. E. and Quamme, G. A. (1984). Mechanisms of CO_2 transport in rectal salt gland of *Aedes*. II. Site of $Cl^-HCO_3^-$ exchange. *J. Exp. Biol.* **246**, R735-R740.

Tashian, R. E., Hewett-Emmett, D., Carter, N. D. and Bergenhem, N. C. H. (2000). Carbonic anhydrase (CA)-related proteins (CA-RPs), and transmembrane proteins with CA or CA-RP domains. In *The Carbonic Anhydrases: New Horizons* (ed. W. R. Chegwidden, N. D. Carter and Y. H. Edwards), pp. 105-120. Basel: Birkhäuser.

Terra, W. R. and Ferreira, C. (1981). The physiological role of the peritrophic membrane and trehalase: digestive enzymes in the midgut and excreta of starved larvae of *Rhynchosciara*. *J. Insect Physiol.* **27**, 325-331.

Terra, W. R., Ferreira, C. and Bianchi, A. G. (1979). Distribution of digestive enzymes among the endo- and ectoperitrophic spaces and midgut cells of *Rhynchosciara* and its physiological significance. *J. Insect Physiol.* **25**, 487-494.

Toyosawa, S., Ogawa, Y., Inagaki, T. and Ijuhin, N. (1996). Immunohistochemical localization of carbonic anhydrase II in rat incisor epithelial cells at various stages of amelogenesis. *Cell Tissue Res.* **285**, 217-225.

Wigglesworth, V. B. (1932). On the function of the so called 'rectal glands' of insects. *J. Cell Sci.* **75**, 131-150.

Zhang, Y. and Frohman, M. A. (1997). Using rapid amplification of cDNA ends (RACE) to obtain full-length cDNAs. *Methods Mol. Biol.* **69**, 61-87.

Zhuang, Z., Linser, P. J. and Harvey, W. R. (1999). Antibody to H^+ V-ATPase subunit E colocalizes with portasomes in alkaline larval midgut of a freshwater mosquito (*Aedes aegypti* L.). *J. Exp. Biol.* **202**, 2449-2460.