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Carbonic anhydrase in the midgut of larval *Aedes aegypti*: cloning, localization and inhibition

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Summary

The larval mosquito midgut exhibits one of the highest pH values known in a biological system. While the pH inside the posterior midgut and gastric caeca ranges between 7.0 and 8.0, the pH inside the anterior midgut is close to 11.0. Alkalization is likely to involve bicarbonate/carbonate ions. These ions are produced *in vivo* by the enzymatic action of carbonic anhydrase. The purpose of this study was to investigate the role of this enzyme in the alkalization mechanism, to establish its presence and localization in the midgut of larval *Aedes aegypti* and to clone and characterize its cDNA. Here, we report the physiological demonstration of the involvement of carbonic anhydrase in midgut alkalization. Histochemistry and *in situ* hybridization showed that the enzyme appears to be localized throughout the midgut, although preferentially in the gastric caeca and posterior regions with specific cellular heterogeneity. Furthermore, we report the cloning and localization of the first carbonic anhydrase from mosquito larval midgut. A cDNA clone from *Aedes aegypti* larval midgut revealed sequence homology to α-carbonic anhydrases from vertebrates. Bioinformatics indicates the presence of at least six carbonic anhydrases or closely related genes in the genome of another dipteran, the fruit fly *Drosophila melanogaster*. Molecular analyses suggest that the larval mosquito may also possess multiple forms.

Key words: carbonic anhydrase, mosquito, *Aedes aegypti*, *Drosophila melanogaster*, larva, midgut, arthropod, alkalization, cDNA, bicarbonate.

Introduction

Mosquitoes belong to the order Diptera, family Culicidae. According to the American Mosquito Control Association, there are more than 2500 different species throughout the world, with 150 species in the United States (Darsie and Morris, 2000; Spielman and D'Antonio, 2001). Mosquitoes act as vectors for a wide variety of diseases such as malaria, yellow fever and dengue fever. Recent reports estimate that fifty to one hundred million cases of dengue fever occur annually, along with several hundred thousand cases of the life-threatening form of the disease dengue hemorrhagic fever (Halstead, 1997). The geographic range of dengue fever has expanded over the last two decades, primarily because of the spread of its principal vector, *Aedes aegypti* (Gubler, 1997). *Aedes aegypti* goes through four larval stages termed instars. In each instar, the larvae possess a series of morphological characteristics, some particular to that stage. However, there are only slight changes in internal organs such as the midgut. The midgut is involved in ionic and osmotic regulation as well as excretion (Clements, 1992). It is subdivided into four structurally distinguishable regions: cardia, gastric caeca, anterior stomach (midgut) and posterior stomach (midgut). Each of these regions consists of an epithelium one cell thick composed of at least two types of cell that vary in character somewhat from region to region.

An interesting and perplexing feature of the mosquito larva midgut is that the luminal compartment exhibits one of the highest pH values known to be generated by a biological system. The pH inside the lumen varies from around 8.0 in the gastric caeca to 11.0 in the anterior midgut to approximately 7.0 in the posterior midgut. The role of the alkaline pH in the anterior midgut is a point of some controversy. It has been suggested that the high pH contributes to the digestion of plant detritus and, in particular, to the dissociation of tannin–protein complexes that ultimately result in enhanced assimilation of proteins (Martin et al., 1980). A detailed model that describes the mechanisms that drive alkalization of the midgut has not been elucidated, but several enzymes have been hypothetically implicated in the process. Among the functions that must be regulated by components of the gut system are the transfer of H^+ and the maintenance of the pH gradient generated by this transfer.

There is strong immunohistochemical (Zhuang et al., 1999)

and physiological (Clark et al., 1999; Boudko et al., 2001b) evidence that an electrogenic, basal H^+ V-ATPase energizes luminal alkalization in the anterior midgut by producing a net extrusion of protons out of the lumen and a hyperpolarization of the basal membrane. In contrast, V-ATPase appears to be localized in the apical membrane of the posterior midgut and gastric caeca, providing a reversed H^+-pumping capacity relative to the anterior midgut (Zhuang et al., 1999). A system capable of generating a high luminal pH is likely to be buffered by CO_3^{2-}, which has a pKa of approximately 10.3. Bicarbonate (and ultimately carbonate) ions are produced *in vivo* primarily by the enzymatic action of carbonic anhydrase. This enzyme catalyzes the reversible hydration/dehydration of carbon dioxide and bicarbonate in most complex organisms. Its activity contributes to the transfer and accumulation of H^+ or HCO_3^- in bacteria, plants, vertebrates and invertebrates. Although there are innumerable reports related to the isolation of carbonic anhydrase from vertebrates, studies involving carbonic anhydrase from invertebrates are very rare, and there are no reports of the isolation of carbonic anhydrase from adult mosquitoes or their larvae.

The purpose of this study was to determine the presence and location of carbonic anhydrase in the midgut of the larva of *Aedes aegypti* and to clone and characterize the enzyme. To investigate the role of carbonic anhydrase in the alkalization of the larval midgut, the effects of carbonic anhydrase inhibitors were tested. Here, we report the cloning and localization of the first carbonic anhydrase from mosquito larvae and, in particular, from the midgut epithelium of larval *Aedes aegypti*. A cDNA clone isolated from fourth-instar *Aedes aegypti* midgut (termed A-CA) revealed sequence homology to the α-carbonic anhydrases (Hewett-Emmett, 2000). Histochemistry and *in situ* hybridization showed that the enzyme appears to be localized throughout the midgut, although preferentially in the gastric caeca and posterior regions. In addition, classic carbonic anhydrase inhibitors such as acetazolamide and methazolamide inhibit the mosquito enzyme in the midgut.

Materials and methods

Experimental insects

Eggs of *Aedes aegypti* were obtained from a colony maintained by the United States Department of Agriculture laboratory (USDA) in Gainesville, FL, USA. The eggs were allowed to hatch in 20 ml of 2 % artificial sea water and reared as described previously (Zhuang et al., 1999; Boudko et al., 2001b).

Preparation and fixation of tissue

To dissect out the midgut, the cold-immobilized larvae were pinned down by the head (using fine stainless-steel pins) to a Sylgard layer at the bottom of a Petri dish. 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 cuticle was gently pulled apart, and the midgut and gastric caeca were removed. In some cases, the gut contents enclosed in the peritrophic membrane slid out, leaving behind the empty midgut. In other cases, it was necessary to remove the peritrophic membrane and its contents manually. For enzyme histochemistry, fixation was in 3 % glutaraldehyde in 0.1 mol l^{-1} phosphate buffer, pH 7.3, overnight at 4 °C (Ridgway and Moffet, 1986). For *in situ* hybridization, dissected tissues were fixed overnight in 4 % paraformaldehyde in 0.1 mol l^{-1} cacodylate buffer (pH 7.2).

In some cases, the dissected larval midguts were photographed using a Nikon FX-35DX camera mounted on a Nikon SMZ-10 dissecting microscope. In other cases, digital images were acquired using a Leica DMR microscope equipped with a Hammamatsu CCD camera. All images were assembled using CorelDraw-9 software.

Bromothymol Blue qualitative assay

A qualitative test to detect carbonic anhydrase activity in mosquito larval midgut homogenates was adapted from the test described by Tashian (1969). The procedure included immersing a piece of Whatman no.1 paper in a solution made with 0.15 % Bromothymol Blue (BTB) in ice-cold 25 mmol l^{-1} Tris-HCl, 0.1 mol l^{-1} Na_2SO_4, pH 8.0. The paper was allowed to soak completely in this blue solution and was placed on ice for 30 min. The colored filter was then transferred to a Petri dish with a hole in the lid. Samples of mosquito larval midgut homogenate were prepared by sonicating midguts of early fourth-instar larvae in ice-cold 25 mmol l^{-1} Tris-HCl, 0.1 mol l^{-1} Na_2SO_4, pH 8.0 containing protease inhibitor cocktail (Sigma-Aldrich; diluted 1:1000). An autopipette was used to spot exactly 4 µl samples on the paper. Controls were also spotted. The controls included a buffer with protease inhibitor and controls for the liver/yeast food added to the medium in which the mosquito larvae were reared. These food controls included a range of concentration from 1 to 100 µg ml^{-1} liver powder and yeast. Carbonic anhydrase from bovine erythrocytes (Sigma-Aldrich) dissolved in the buffer described above was used as an additional control.

A steady stream of CO_2 at 34.5 kPa was blown for 3 s through the opening on the lid of the Petri dish, and the dish was sealed and kept on ice. The formation of yellow spots in a few seconds was indicative of carbonic anhydrase activity.

Effect of methazolamide on the alkalization of the midgut of live larvae

The effect of a carbonic anhydrase inhibitor (methazolamide) on gut alkalization and the capacity of whole larvae to alkalize their culture medium was examined. Flat-bottomed 24-well tissue culture plates (Sarstedt, Inc.) were filled with 1 ml of 25 mmol l^{-1} Tris-HCl, 0.1 mol l^{-1} Na_2SO_4 buffer, pH 8.5. BTB solution was added to each well to a final concentration of 0.003 %. Five live early fourth-instar larvae that had been placed in BTB indicator solution for 2 h were added to each of the wells, and the larvae were allowed to adjust to their new environment for 30 min. Methazolamide dissolved in dimethyl sulfoxide (DMSO) at concentrations ranging from 10^{-6} to 8×10^{-3} mol l^{-1} was added to the wells. Controls included

DMSO with indicator and no inhibitor and controls with buffer, indicator and no DMSO. The plates were scanned, using a Hewlett Packard ScanJet 6100C scanner, before addition of the inhibitor and 5 h later. In addition, the midguts were dissected and photographed to record the pH within the gut lumen as revealed by the color of ingested BTB.

^{18}O-exchange method to measure carbonic anhydrase activity

Tissue homogenate carbonic anhydrase activity was measured using the ^{18}O-exchange method (Silverman and Tu, 1986). Midguts were dissected, and the peritrophic membrane was removed together with its contents. Individual measurements of carbonic anhydrase activity were performed with pooled samples of gastric caeca, anterior midgut, posterior midgut and Malpighian tubules. The method involved adding ^{18}O-labeled $NaHCO_3$ to 0.1 mol l^{-1} Hepes buffer, pH 7.6, at 9.5 °C. The disappearance of ^{18}O from CO_2 and/or HCO_3^- upon addition of the enzyme preparations was monitored. Measurements of ^{18}O in CO_2 were accomplished with a mass spectrometer, using a CO_2-permeable inlet that allowed very rapid, continuous measurement of the isotopic content of CO_2 in solution. All samples were centrifuged at 14 000 revs min^{-1} at room temperature (24–25 °C) prior to the assay to remove food and insoluble material. Inhibition was accomplished by adding methazolamide to a final concentration of 10^{-6} mol l^{-1}.

Isolation of RNA and synthesis of cDNA

Total RNA was isolated from freshly dissected mosquito larval midguts using TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH, USA) according to the manufacturer's instructions. Briefly, 100 *A. aegypti* gut epithelial organs, including fore-, mid- and hindgut (approximately 20 mg), were dissected into 200 µl of ice-cold TRI Reagent and homogenized in a sterile microcentrifuge tube. TRI Reagent (600 µl) was added to the homogenate and incubated for 5 min at room temperature. The homogenate was then extracted with chloroform and precipitated with isopropanol. The RNA pellet was washed with 75 % ethanol, dried in air and resuspended in diethylpyrocarbonate-treated water. RNA concentrations were calculated from the absorbance at 260 nm.

Total RNA (10 µg) was reverse-transcribed in a 20 µl reaction mixture using 5 pmol of oligo(dT)12-18 and 200 units of Superscript II RNase H-reverse transcriptase (Life Technologies, Inc., Grand Island, NY, USA), according to the manufacturer's instructions.

Cloning of carbonic anhydrase from mosquito larval midgut

Degenerate oligonucleotides were designed for the regions of conserved amino acids among carbonic anhydrase proteins as determined by BLAST analysis of several vertebrate and two putative, but annotated, carbonic anhydrases from the *Drosophila melanogaster* sequence data base.

The primer sequences used initially were: CA5F, 5′-GARCARTTYCAYTKY CAYTGGGG-3′; and CA3R, 5′-GTIARISWNCCYTCRTA-3′, where *N*=G, A, T or C, K=G or T, S=G or C, W=A or T, Y=C or T and R=A or G. PCR reactions were performed in a total volume of 20 µl, and the reaction mixture contained 0.1 µg of cDNA as template, 0.2 µmol l^{-1} of each primer, 200 µmol l^{-1} each of deoxynucleotidyl triphosphates, 1× PCR buffer and 1 unit of *Taq* polymerase (Clontech). The PCR cycling profile was: 94 °C for 5 min, 55 °C for 2 min and 72 °C for 3 min, followed by six cycles of 94 °C for 0.5 min, 53 °C (in increments of 2 °C per cycle) for 1 min and 72 °C for 1 min and 35 cycles of 94 °C for 0.5 min, 45 °C for 1 min and 72 °C for 2 min followed by a final extension at 72 °C for 15 min. The PCR products were visualized on 1 % agarose gels, and specific products were gel-extracted (Qiagen, Inc, Valencia, CA, USA), diluted 1:100 in water and used as template for a second, identical PCR. The resulting 297-base-pair (bp) product was gel-purified, ligated into pGem-T and transformed into JM109 *Escherichia coli* for subcloning.

3′ and 5′ rapid amplification of cDNA ends

The cDNA was extended into the 3′-and 5′-untranslated regions (UTRs) by rapid amplification of cDNA ends (RACE), modified from the method of Zhang and Frohman (1997). Exact primers were then defined on the basis of UTRs. Reverse-transcriptase/polymerase chain reaction (RT-PCR) was then used to produce a single product whose consensus start and stop codons bracket 894 nucleotides encoding a 298-residue polypeptide.

Sequencing

Plasmid DNA from individual colonies was purified using a Qiaprep Plasmid Mini kit (Qiagen Inc., Valencia, CA, USA). The plasmid DNA was then 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.

In situ *hybridization*

Sense and antisense digoxygenin (DIG)-labeled carbonic anhydrase cRNA probes were generated by *in vitro* transcription of the original 297 bp A-CA cDNA according to the manufacturer's protocols (Roche Molecular Biochemicals, Indianapolis, IN, USA).

For *in situ* hybridization, methods were adapted from Westerfield (1994). Briefly, the midguts of fourth-instar *A. aegypti* larvae were dissected free from surrounding tissue in hemolymph substitute solution (HSS) (Clark et al., 1999) consisting of 42.5 mmol l^{-1} NaCl, 3.0 mmol l^{-1} KCl, 0.6 mmol l^{-1} $MgSO_4$, 5.0 mmol l^{-1} $CaCl_2$, 5.0 mmol l^{-1} $NaHCO_3$, 5.0 mmol l^{-1} L-succinic acid, 5.0 mmol l^{-1} L-malic acid, 5.0 mmol l^{-1} L-proline, 9.1 mmol l^{-1} L-glutamine, 8.7 mmol l^{-1} L-histidine, 3.3 mmol l^{-1} L-arginine, 10.0 mmol l^{-1} dextrose, 25 mmol l^{-1} Hepes, pH 7.0 adjusted with NaOH. The guts were immediately fixed with 4 % paraformaldehyde in 0.1 mol l^{-1} sodium cacodylate buffer overnight at 4 °C. The midguts were washed with PBS at room temperature and then incubated in 100 % methanol at –20 °C for 30 min to ensure permeabilization of the gut tissue. The tissue was washed

(5 min each wash) in 50 % methanol in PBST [Dulbecco's phosphate-buffered saline (Sigma) plus 0.1 % Tween-20], followed by 30 % methanol in PBST and then PBST alone. The tissue was fixed in 4 % paraformaldehyde in 0.1 mol l^{-1} sodium cacodylate buffer for 20 min at room temperature and washed with PBST. The larval midguts were digested with proteinase K (10 μg ml^{-1} in PBST) at room temperature for 10 min, washed briefly with PBST and fixed again, as described above.

Prehybridization of the tissue was accomplished by incubation in HYB solution [50 % formamide, 5× SSC (1× SSC is 0.15 mol l^{-1} NaCl, 0.015 mol l^{-1} sodium citrate buffer, pH 7.0), 0.1 % Tween-20] for 24 h at 55 °C. The larval midguts were transferred to HYB+ solution (HYB plus 5 mg ml^{-1} tRNA, 50 μg ml^{-1} heparin) containing 5 ng ml^{-1} DIG-labeled probe and incubated overnight at 55 °C. Excess probe was removed by washing at 55 °C with 50 % formamide in 2× SSCT for 30 min (twice), 2× SSCT for 15 min and 0.2× SSCT for 30 min (twice). For detection, the tissue was incubated in PBST containing 1 % blocking solution (Roche Molecular Biochemicals) for 1 h at room temperature. The tissue was incubated with anti-DIG alkaline phosphatase (Roche Molecular Biochemicals) diluted 1:5000 in blocking solution for 4 h at room temperature. The tissue was washed with PBST and incubated in alkaline phosphatase substrate solution (Bio Rad Laboratories, Hercules, CA, USA) until the desired intensity of staining was achieved.

Histochemistry

Carbonic anhydrase activity was detected in isolated *A. aegypti* midgut using Hansson's method (Hansson, 1967) as modified by Ridgway and Moffet (1986). Briefly, the procedure involved incubation of isolated, glutaraldehyde-fixed midguts in 1.75×10^{-3} mol l^{-1} $CoSO_4$, 5.3×10^{-2} mol l^{-1} H_2SO_4, 11.7×10^{-3} mol l^{-1} KH_2PO_4 and 1.57×10^{-2} mol l^{-1} $NaHCO_3$ (pH 6.8). The cobalt precipitate in the midguts was visualized using 0.5 % $(NH_4)_2S$ in distilled water. Removal of the substrate ($NaHCO_3$) eliminated staining.

Results

Establishing the presence of carbonic anhydrase in Aedes aegypti *larvae*

The presence of carbonic anhydrase in the midgut of larval *Aedes aegypti* has been confirmed using direct methods, such as Hansson's histochemical stain, the ^{18}O-exchange method and *in situ* hybridization, as well as indirect methods, such as the BTB assay and inhibition with carbonic-anhydrase-specific inhibitors. The enzyme in the midgut is most strongly associated with the gastric caeca and the posterior portion of the midgut.

Bromothymol Blue qualitative assay

This assay allowed the identification of samples of solubilized midgut tissue containing carbonic anhydrase activity by spotting them onto a filter paper soaked in a basic buffered solution containing a pH indicator (BTB). As stated previously, BTB changes color from yellow at pH<7.6 to blue when the pH increases above this value. This assay is based on the principle that carbonic anhydrase catalyzes the conversion of CO_2 into bicarbonate with the concomitant release of protons (Donaldson and Quinn, 1974). The presence of protons lowers the pH in those regions of the paper where the spotted samples contain the enzyme. As the pH falls below 7.6, these spots rapidly change color from blue to yellow. This assay is not effective for samples in acidic solution, and the tissue homogenization must be accomplished in alkaline buffer. The enzymatic reaction takes only a few seconds, and it can be delayed if the solutions, the paper and the samples are kept cold on ice. However, a few seconds is usually sufficient to discriminate the samples that contain carbonic anhydrase from those lacking enzymatic activity. The assay must be performed quickly since, after approximately 1 min, the entire filter paper turns yellow, probably as a result of the uncatalyzed hydration of carbon dioxide absorbed by the solution at this basic pH.

The test has proved useful in determining the presence of small amounts of carbonic anhydrase in homogenates of mosquito larvae. The assay was also useful for detecting carbonic anhydrase activity qualitatively in fractions obtained from affinity chromatography (Osborne and Tashian, 1975) of larval homogenates. The affinity chromatographic procedure, which employs a bound carbonic anhydrase inhibitor, *p*-aminomethyl benzyl sulfonamide (*p*-AMBS; Sigma), produced two peaks of carbonic anhydrase activity upon exposure to the standard elution buffers. The amount of protein that we were able to produce by this technique was, however, very small and resisted several efforts at direct microsequencing. This change in color was inhibited by acetazolamide and methazolamide when these inhibitors (10^{-5} mol l^{-1}) were added to the samples prior to spotting on the dye-impregnated filter papers. Inhibition of the reaction resulted in blue spots that did not change color upon addition of CO_2. The positive control containing commercial carbonic anhydrase turned yellow when carbon dioxide was added, and this color change was also inhibited by acetazolamide and methazolamide. This finding confirmed that the yellow color of the spots was due to the action of carbonic anhydrase and that the mosquito larva contains active carbonic anhydrase.

Carbonic anhydrase activity and alkalization

The classic and specific carbonic anhydrase inhibitor methazolamide was tested in live fourth-instar larvae to examine the influence of carbonic anhydrase on the maintenance of the pH extremes inside the midgut and the effect of the enzyme on the net alkalization of the growth medium by the intact animals. Previous investigations have shown that living mosquito larvae excrete bicarbonate, which results in the net alkalization of their surrounding aqueous medium (Stobbart, 1971). Equal numbers of living larvae of equivalent age and size were placed in culture plate wells containing lightly buffered medium and the pH indicator BTB. The tissue culture plates used in this assay were scanned before and after addition of various concentrations of

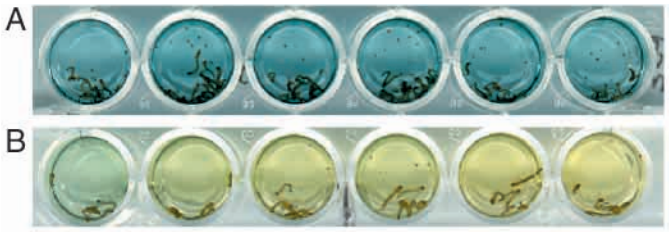

Fig. 1. Effect of inhibition of carbonic anhydrase on the pH of culture medium of fourth-instar larvae of *Aedes aegypti*. Mosquito larvae typically alkalize the medium in which they are reared (Stobbart, 1971). (A) Six culture wells each containing five fourth-instar larvae incubated for 5 h in medium containing 0.003 % Bromothymol Blue. The blue color is retained, indicating a pH greater than 7.6. (B) The same as A, except that each well also contains a different concentration of the specific carbonic anhydrase inhibitor methazolamide ranging from 10^{-6} to 10^{-3} mol l^{-1} from left to right. A yellow color indicates a pH below 7.6.

methazolamide. In the absence of methazolamide, the blue color of the medium, indicating a pH of at least 7.6, was maintained (Stobbart, 1971). Actual measurement of the pH in each well showed a slow increase over time (data not shown). Upon addition of methazolamide, the culture medium slowly became acidic, with a resulting change in color to yellow as the pH dropped below 7.6 (Fig. 1). All the controls that did not contain methazolamide remained blue. Addition of methazolamide, at the various concentrations used here, to culture plate wells containing only medium with BTB (no mosquito larva control) had no effect on their color; they remained blue. These data show that carbonic anhydrase activity is present in the living larvae and that it plays some role in acid–base excretion.

Moreover, fourth-instar larvae cultured in BTB-containing medium ingest the dye, which can then be used as a visible indicator of the pH of the gut lumen. Treatment of the cultured larvae with methazolamide showed a direct impact of carbonic anhydrase activity on gut luminal pH. Fig. 2 compares the luminal pH of dissected larval midguts with and without a 5 h exposure to methazolamide. The micrographs reveal that alkalization of the midgut was inhibited by methazolamide as shown by the color change of the BTB indicator. Interestingly, the effect was most pronounced in the anterior midgut, where the pH indicator changed from blue in the midgut of larvae reared in the absence of inhibitor to yellow in as little as 30 min when methazolamide (10^{-6} mol l^{-1}) was added to the culture. The indicator also changed color progressively from blue through green to yellow in the gastric caeca (Fig. 2). No apparent change was observed in the posterior midgut. The color of the midgut in this region was yellow both in the untreated larvae and in the larvae treated with methazolamide. Since the pH of the posterior midgut has been associated with values close to 7.6, no change in color was evident using this qualitative method.

^{18}O isotope-exchange experiments

The relative activity of carbonic anhydrase, normalized to total protein content, was calculated as described by Silverman and Tu (1986). The relative activity of carbonic anhydrase was highest in the gastric caeca, followed by the posterior midgut and Malpighian tubules, as shown in Fig. 3. The relative activity of carbonic anhydrase in the anterior midgut was either extremely low or non-existent, falling at or below that of the buffer blank. The specificity of the reaction was confirmed by its complete inhibition by the addition of 10^{-6} mol l^{-1} methazolamide (results not shown).

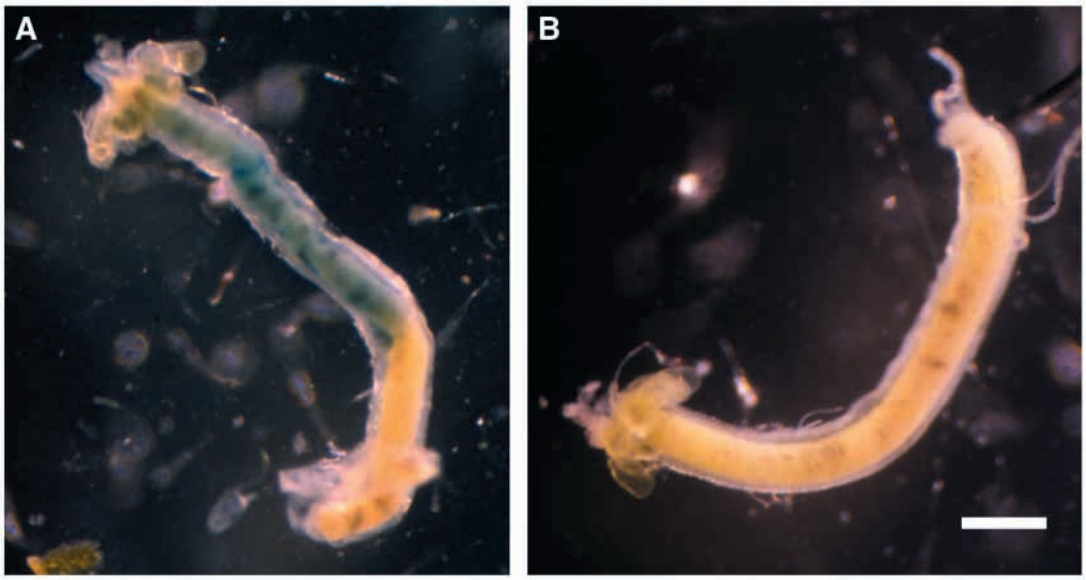

Fig. 2. Effect of methazolamide on the alkalization of the midgut using the Bromothymol Blue (BTB) assay of pH within living, but isolated, gut tissue. Gut tubes were dissected after pre-loading with BTB and then incubated for 5 h in hemolymph substitute (Clark et al., 1999) in the absence (A) or presence (B) of 10^{-6} mol l^{-1} methazolamide. The loss of blue coloration in B shows that the internal pH of the gut lumen has dropped below 7.6. Scale bar, 600 μm.

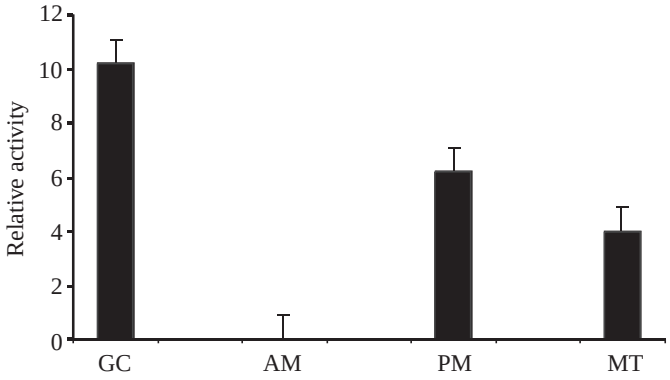

Fig. 3. Relative activity of carbonic anhydrase in different pooled segments of the midgut of larval *Aedes aegypti*. Midguts were dissected from early fourth-instar larvae and separated into gastric caeca (GC), anterior midgut (AM), posterior midgut (PM) and Malpighian tubules (MT). The relative activity of carbonic anhydrase was measured using the O^{18} mass spectrometry method (Silverman and Tu, 1986) normalized to total protein content. The activity of the anterior midgut was lower than that of the water blank and, thus, is set as 'zero' activity. Values are means + S.E.M., N=3.

Cloning of carbonic anhydrase from Aedes aegypti *larvae*

Attempts to isolate and purify carbonic anhydrase using *p*-AMBS linked to agarose proved successful. However, sequencing the resulting proteins was not. Therefore, we utilized other molecular biological techniques to clone a specific carbonic anhydrase cDNA from the epithelial cells of the midgut of larval mosquitoes. A comparison of 12 carbonic anhydrase sequences, including two putative sequences that had been so annotated but not characterized in the *Drosophila melanogaster* data bases, was made.

We produced degenerate PCR primers from consensus regions of the carbonic anhydrase gene family. The generation of an initial 297 bp partial sequence from which we derived exact PCR primers for 3′- and 5′-RACE (Zhang and Frohman, 1997), facilitated the eventual cloning of a single contiguous cDNA. The final contiguous region spanned both the start and stop codons and encoded a polypeptide of 298 residues (sequence submitted to GenBank, accession number AF395662). Fig. 4A shows an alignment of the *A. aegypti* carbonic anhydrase (A-CA) amino acid sequence with several other previously characterized members of this extensive gene family. Fig. 4B shows the homology tree generated using DNAman software. Fig. 5A shows the alignment between A-CA and six putative carbonic anhydrase gene sequences from the *Drosophila melanogaster* genome that our homology search (BLAST) revealed. Four of the *Drosophila melanogaster* genes (AAF54494, AAF56666, AAF57140 and AAF57141) had not previously been annotated. Fig. 5B shows the homology tree generated with these sequences. A-CA has a putative molecular mass of 32.7 kDa. Hydrophobicity analysis suggests that this protein is a typical soluble enzyme and possesses a characteristic eukaryotic-type carbonic anhydrase signature sequence within the polypeptide (amino acid residues 99–115) (Fernley, 1988).

To examine the possibility of regionalized expression of the A-CA, PCR using exact primers was performed on RNA samples harvested from the various sections of the gut. Fig. 6 shows an ethidium-bromide-stained agarose gel. PCR products of the expected 894-nucleotide length are readily seen in the RNA isolated from the gastric caeca and the posterior midgut. The anal papillae (not shown), anterior midgut, Malpighian tubules and rectal gland showed little or no PCR product. This result is consistent with the relative enzyme activities described above. When the material was subjected to a second round of PCR using the same primers, an appropriately sized product was discernible in the Malpighian tubules and the anterior midgut. These results suggest that A-CA may be expressed throughout the gut, but at much higher levels in the caeca and the posterior midgut than in other regions. In addition, this PCR analysis revealed higher-molecular-mass products in the anterior midgut and Malpighian tubules that may represent additional carbonic anhydrases specific to larval mosquito (Fig. 6).

Localization of the enzyme in the midgut epithelium: carbonic anhydrase enzyme histochemistry

To analyze further the regional and cellular expression of carbonic anhydrase in the midgut epithelium of larval mosquitoes, a modified Hansson's histochemical reaction was performed on whole-mount preparations of the gut (Hansson, 1967). Fig. 7 summarizes the results of this analysis. Carbonic anhydrase activity was detected in a non-uniform pattern along the length of the gut. The most intense staining was evident in the gastric caeca and the posterior midgut. Staining was less intense in the anterior midgut. At higher magnification, it was obvious that cellular heterogeneity with regard to carbonic anhydrase activity also exists. This is particularly evident in the posterior midgut, where very large and regularly spaced cells appear nearly white on a background of dark carbonic anhydrase reaction product. The larger cells have been characterized as 'columnar' or ion-transporting cells (Volkmann and Peters, 1989b). Surrounding these large cells are more numerous smaller cells termed 'cuboidal' or resorbing/secreting cells (Zhuang et al., 1999). The carbonic anhydrase histochemical stain clearly distinguishes these cell types from one another and indicates that the large columnar cells contain very little carbonic anhydrase in comparison with the smaller cuboidal cells. In addition, the posterior-most cells of each lobe of the gastric caeca show little or no histochemical staining, suggesting further cellular heterogeneity with respect to carbonic anhydrase distribution in the midgut (Fig. 7).

In situ *hybridization*

To characterize further the localization of A-CA expression, *in situ* hybridization was performed using a portion (approximately 300 bp) of the central coding region of the cDNA. Fig. 8 shows results typical of this type of analysis. A strong hybridization signal was evident in the gastric caeca and the posterior midgut. Lower levels of hybridization were evident in other gut regions. As with the histochemical stain,

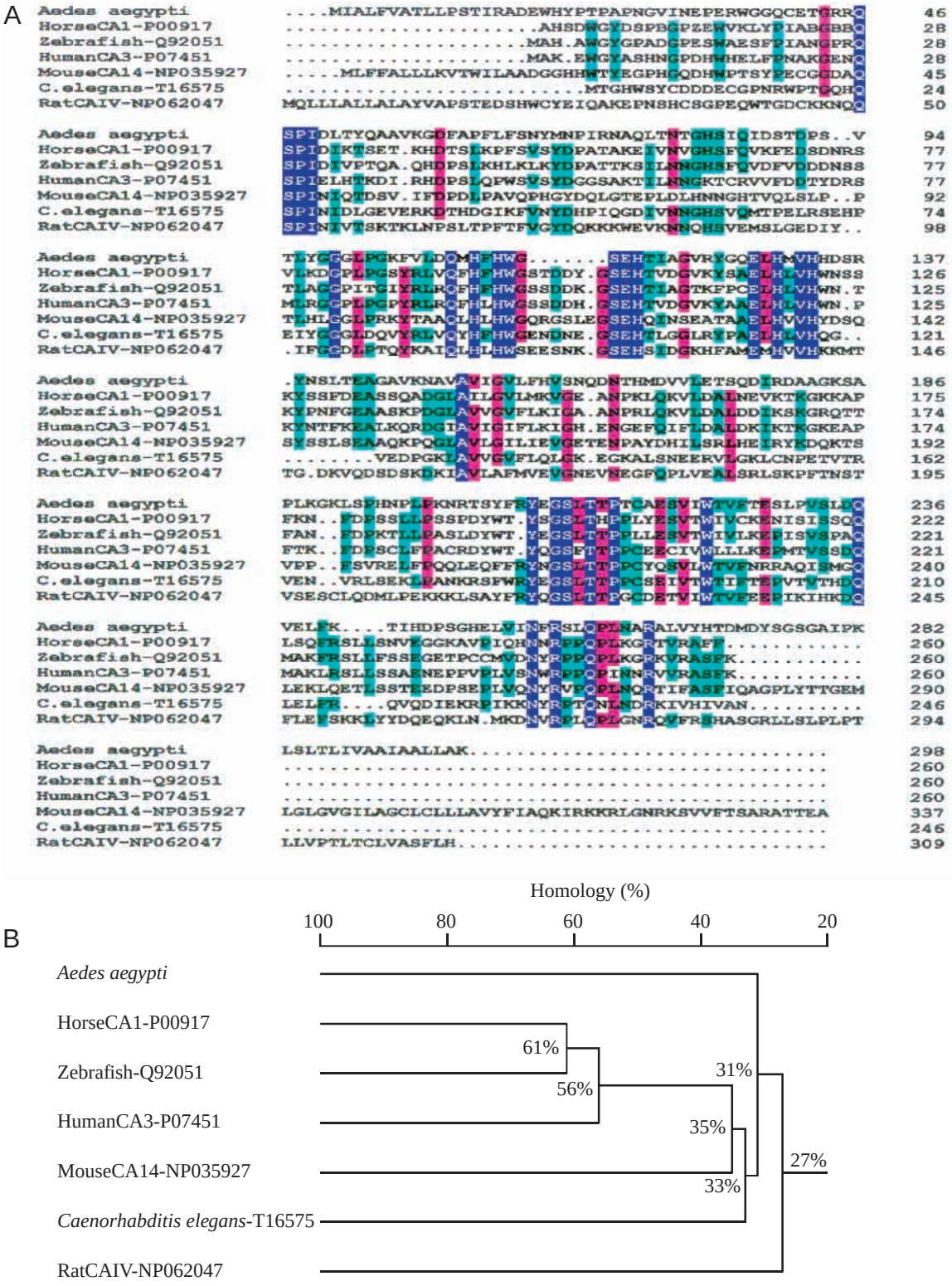

Fig. 4. Carbonic anhydrase from the midgut of larval *Aedes aegypti*. (A) Alignment (BLAST) of the predicted amino acid sequence of *Aedes aegypti* cDNA (A-CA) (GenBank accession number AF395662) with several known α-carbonic anhydrases. Regions of exact homology across all species are highlighted in blue (100 %); regions with less homology are highlighted in red (>75 %) and green (>50 %). Similar amino acid residues have been included in the shading. (B) A homology tree comparing A-CA and several vertebrate α-carbonic anhydrases (DNAman software).

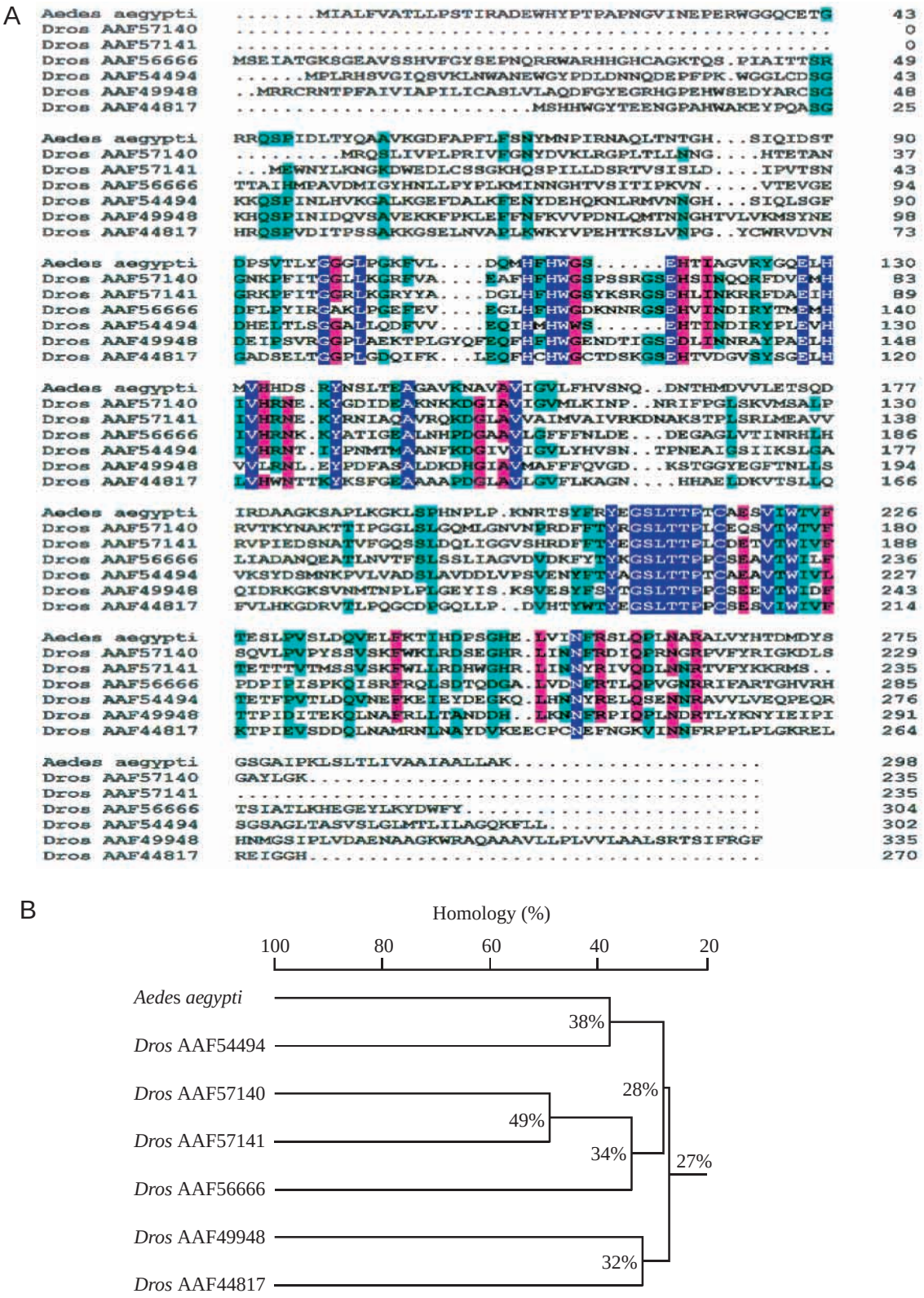

Fig. 5. Comparison of the extrapolated amino acid sequences of A-CA with six putative dipteran carbonic anhydrase genes identified in the *Drosophila melanogaster* gene data bases. (A) An alignment of A-CA with the amino acid sequences of the six *Drosophila melanogaster* genes (with accession numbers listed) identified through bioinformatics searching. Regions of exact homology across all species are highlighted in blue (100 %); regions with less homology are highlighted in red (>75 %) and green (>50 %). Similar amino acid residues were included in the shading. (B) A homology tree comparison of these dipteran carbonic anhydrases.

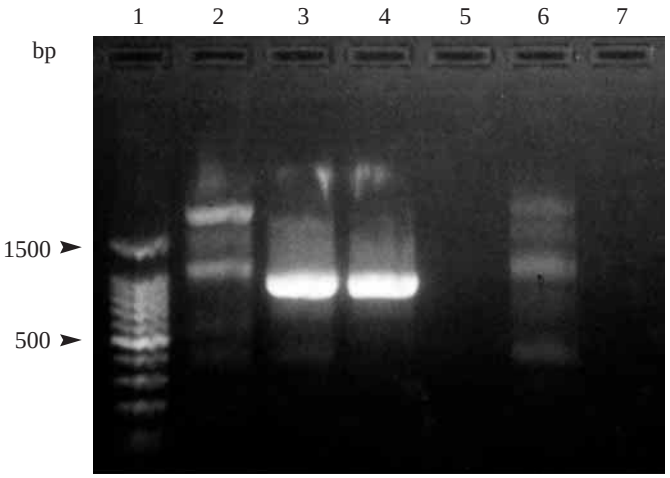

Fig. 6. Polymerase chain reaction (PCR) analysis of *Aedes aegypti* cDNA (A-CA)-like sequences in cDNA-amplified RNA samples taken from regions of the midgut of larval *Aedes aegypti*. PCR was performed using exact primers for the cloned A-CA. Equal quantities of total RNA were amplified and analyzed from anterior midgut (lane 2), gastric caeca (lane 3), posterior midgut (lane 4), whole gut (lane 5), Malpighian tubules (lane 6) and a water template control (lane 7). Note the primary product in caeca and posterior midgut samples at the expected size of approximately 894 nucleotides. Also note the absence of this band from other gut regions but the appearance of bands of higher molecular masses. Lane 1 is a 100 base pair (bp) molecular mass ladder.

higher magnification revealed that the relatively small cuboidal cells exhibited more intense labeling than did the large columnar cells (Fig. 8).

Discussion

Carbonic anhydrase was first characterized in erythrocytes as the result of a search for a catalytic factor that would enhance the transfer of bicarbonate from the erythrocyte to the pulmonary capillaries (Meldrum and Roughton, 1933). Since then, the enzyme has been shown to play an important role in most acid/base-transporting epithelia. The search for the enzyme in the midgut of the larval mosquito was triggered by the observations of a pH value around 11 in the anterior midgut lumen and a high bicarbonate concentration (e.g. Zhuang et al., 1999; Boudko et al., 2001b).

The presence of carbonic anhydrase in the midgut of the larval mosquito has been suggested before by investigations of the epithelium of larval lepidopteran midgut. Carbonic anhydrase has been studied in *Manduca sexta*, where the enzyme has been associated with the fat body, midgut and integumentary epithelium (Jungreis et al., 1981). The enzyme has also been localized in the goblet cells of the epithelium of *Hyalophora cecropia* using Hansson's histochemical stain. The same procedure showed that the columnar cells were devoid of activity (Turbeck and Foder, 1970).

Even though a number of genes and their products have been isolated from the midgut of *Aedes aegypti*, and a role for carbonic anhydrase in the alkalization of the midgut has been suggested (Turbeck and Foder, 1970; Haskell et al., 1965; Ridgway and Moffett, 1986; Boudko et al., 2001b), there have been no reports of the isolation or cloning of carbonic anhydrase or of the localization of the enzyme within the midgut of larval mosquitoes.

Our results show that at least one (and perhaps more) carbonic anhydrase is present in the midgut of larval *Aedes aegypti*. The carbonic anhydrase of larval *Aedes aegypti* (A-CA) is inhibited by classical carbonic anhydrase inhibitors such as methazolamide and acetazolamide. Methazolamide has the most potent effect on A-CA. Direct physiological

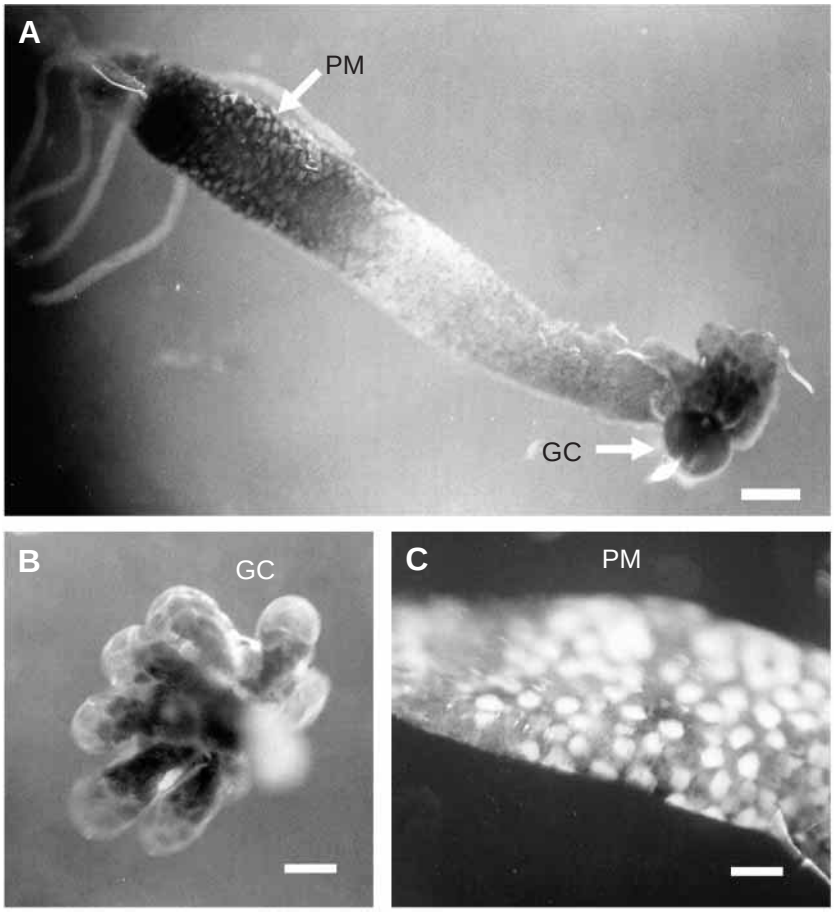

Fig. 7. Hansson's histochemistry of whole, dissected midgut from early fourth- instar larvae of *Aedes aegypti* viewed at low (A) and high (B,C) magnification. Intense dark staining is observed in the gastric caeca (GC) and the posterior midgut (PM), indicating carbonic anhydrase activity. Large, relatively unstained cells are also evident in the posterior midgut (A,C). The posterior-most extensions of the lobes of the gastric caeca exhibit relatively low levels of reaction product, indicating lower levels of the enzyme in these cells relative to other cells of the caeaca. Scale bars: 350 μm (A), 250 μm (B), 200 μm (C).

measurements of ion fluxes from living larval mosquito midgut epithelial cells also show methazolamide to be a very potent inhibitor of ion movements and balance (Boudko et al., 2001a).

In spite of the fact that isolation of the enzyme using conventional techniques for protein purification did not yield a microsequenceable protein, a carbonic anhydrase cDNA was cloned from larval midgut. This is the first recorded cloning of a mosquito carbonic anhydrase and, indeed, it is the first to be cloned from any arthropod. Two gene sequences with significant homology to eukaryotic α-carbonic anhydrases were previously annotated in the *Drosophila melanogaster* sequence data base. These sequences were used in our cloning strategy. Our subsequent analyses of the mosquito carbonic anhydrase revealed that four additional carbonic-anhydrase-like gene sequences are readily detected in the *Drosophila melanogaster* genome data base. Thus, it appears that the fruit fly possesses multiple carbonic anhydrase genes, perhaps even more than the six that we have addressed in this paper. The mammalian genome possesses at least 11 carbonic anhydrase genes (Hewett-Emmett, 2000), so the existence of multiple forms in dipterans is perhaps not surprising. Our PCR analysis (Fig. 6) suggests that *Aedes aegypti* also possesses more than one carbonic anhydrase, and we are now attempting to isolate additional isoforms.

To investigate the distribution of carbonic anhydrase in the midgut of the larval mosquito, we employed both *in situ* hybridization and enzyme histochemistry. Our results indicated that enzymatic activity was greatest in the gastric caeca and the posterior midgut, as demonstrated by the intense staining obtained using Hansson's method and by *in situ* hybridization using cRNA probes. Measurements of activity using the O^{18}-exchange method in pools of dissected regions of the gut corroborated these findings. In addition, the enzyme seems to be preferentially associated with the small cuboidal cells in the midgut epithelium, as determined both by enzyme histochemistry and by *in situ* hybridization.

As reviewed by Clements (1992), two major cell types have been defined in the gastric caeca by inferring functional states from cytological findings. These two major cell types have been called ion-transporting cells and resorbing/secreting cells (Volkmann and Peters, 1989a,b) and they correspond to the columnar and cuboidal cells mentioned above, with the ion-transporting cells being equivalent to the columnar cells and the resorbing/secreting cells being the cuboidal cells (Zhuang et al., 1999). Neither of these cell types, as characterized in the larval mosquito gut, parallels the structurally unique qualities of the lepidopteran goblet cell. Nonetheless, our results indicate that, as in lepidopterans, carbonic anhydrase activity is preferentially associated with one of two distinct cell types whose functional complementation must produce the alkalization and ionic balances regulated by the gut. These results are consistent with the observations of lepidopteran midgut by Turbeck and Foder (1970). In the larval lepidopteran midgut, two morphologically distinct cell types have long been recognized: goblet cells and columnar cells. Goblet cells possess both the proton-pumping V-ATPase and carbonic anhydrase activity (Harvey, 1992; Ridgway and Moffet, 1986; Wieczorek et al., 1999). One of the enigmas of using the pioneering analyses of insect model systems such as *Manduca sexta* to produce testable hypotheses for gut alkalization in mosquito larvae has been the apparent absence of goblet cells from mosquitoes. Previous investigations have inferred different functional cell types in the larval mosquito gut epithelium. We are currently developing antibody probes for A-CA. Immunocytochemical analyses of A-CA distribution in comparison with other key components of gut function, such as V-ATPase (Zhuang et al., 1999), should provide new insights into the cell biology of this intriguing epithelial system.

It is interesting to note that the lowest concentration of carbonic anhydrase in the midgut epithelium occurs in the region that surrounds and probably regulates the region of highest luminal pH, the anterior midgut. The pKa of CO_3^{2-} is approximately 10.3 and, hence, this anion is likely to be the

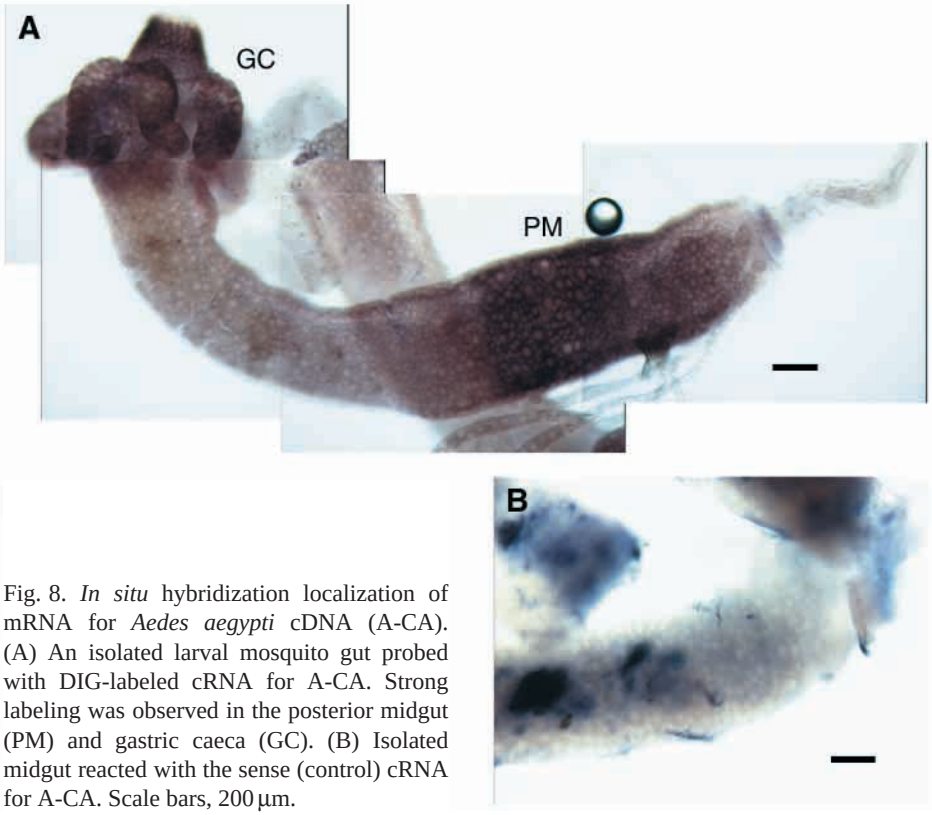

Fig. 8. *In situ* hybridization localization of mRNA for *Aedes aegypti* cDNA (A-CA). (A) An isolated larval mosquito gut probed with DIG-labeled cRNA for A-CA. Strong labeling was observed in the posterior midgut (PM) and gastric caeca (GC). (B) Isolated midgut reacted with the sense (control) cRNA for A-CA. Scale bars, 200 μm.

primary buffer of the pH 10.5–11 gut contents in the anterior midgut. Our results therefore suggest that the major buffering anion in this area of the midgut is probably not produced by local carbonic anhydrase but instead either upstream, in the gastric caeca, or downstream, in the posterior midgut, where carbonic anhydrase levels are very high. This result, and results presented elsewhere (Boudko et al., 2001a), are consistent with a model in which a major function of the anterior midgut is to pump protons out of this region of the gut lumen, promoting the conversion of HCO_3^- to CO_3^{2-}. A comprehensive model of the regulation of ion homeostasis and gut alkalization in the larval mosquito awaits the characterization and localization of other major components of the system in addition to carbonic anhydrase. It will also be very important to resolve the question of whether multiple carbonic anhydrases are expressed in the midgut and how each is distributed in this dynamic tissue.

Quantitative evidence corroborating the distribution of carbonic anhydrase within the midgut and supporting the histochemical and *in situ* observations was obtained using the ^{18}O-exchange mass spectrometric method. The results obtained with this method indicate that the gastric caeca exhibit the highest level of carbonic anhydrase, relative to total protein content, followed by the posterior midgut and the Malpighian tubules. The anterior midgut showed levels of activity so low that two possibilities could be considered: either the method could not detect the enzyme or it is absent from the anterior midgut. The presence of faint staining using the histochemical and *in situ* methods suggests that the levels of activity in the anterior midgut might be too low to be detected using the ^{18}O-exchange method, but that the enzyme is present throughout the entire length of the midgut.

In summary, our evidence demonstrates the existence of carbonic anhydrase in *Aedes aegypti* larvae and it also suggests that the gastric caeca and posterior midgut exhibit the highest levels of carbonic anhydrase activity. In addition, the enzyme seems to be associated with the small cuboidal cells of the midgut epithelium. Our cDNA sequence evidence also suggests that carbonic anhydrase is a soluble, cytosolic enzyme. However, enzyme activity has also been detected in membrane preparations isolated from whole midguts and could be due to the presence of more than one isoenzyme (M. P. Corena and P. J. Linser, unpublished observations). Carbonic anhydrase activity has previously been demonstrated in the epithelium of the larval midgut of six species of lepidopteran, in which it has been associated with the particulate fractions of the homogenate (Turbeck and Foder, 1970). This is consistent with our hypothesis that there might be more than one carbonic anhydrase and that one of these enzymes may be associated with the plasma membrane.

What is the role of carbonic anhydrase in the alkalization mechanism? BTB proved useful in monitoring the impact of carbonic anhydrase inhibition on the maintenance of gut luminal pH and the excretion of acid–base equivalents. As mentioned above, *Aedes aegypti* larvae typically alkalize the medium in which they are reared by secreting bicarbonate (Stobbart, 1971). The ingestion of carbonic anhydrase inhibitors altered the metabolism of the larvae to the point that the metabolic products secreted into the medium changed the pH of the environment, shifting it towards more acidic values than those observed in the absence of inhibitors.

The lowering of the pH of the medium might be related to a decrease in the rate of secretion of HCO_3^-. The effect of the ingestion of carbonic anhydrase inhibitors on the secretion of bicarbonate into the medium remains to be explored. However, as indicated by measurements with ion-selective microelectrodes, inhibition of carbonic anhydrase in the midgut has an extreme effect on the maintenance of an alkaline pH within the midgut lumen (Boudko et al., 2001a). It is plausible that a decrease in the rate of secretion of bicarbonate is elicited by inhibiting the enzyme.

A simple model of bicarbonate transport fails to explain how the high pH is achieved within the mosquito larval anterior midgut. At a pH of approximately 11, similar to that observed within the anterior midgut, the majority of bicarbonate is present as carbonate. In fact, measurements of lepidopteran midgut fluid have shown that it contains 37 mmol l^{-1} carbonate and 17 mmol l^{-1} bicarbonate (Turbeck and Foder, 1970). Since the pH of a 0.1 mol l^{-1} solution of sodium bicarbonate is only approximately 8.3, secretion of bicarbonate alone cannot be responsible for the high pH observed in the anterior midgut (Dow, 1984). It could, however, explain the pH values at the gastric caeca and posterior midgut. The mechanism for maintenance of an alkaline pH within the anterior midgut must be more complex than just a simple buffering of a physiological solution with bicarbonate. Although this mechanism has been investigated (Wieczorek et al., 1999; Boudko et al., 2001a; Zhuang et al., 1999), its details remain unclear. However, the evidence suggests that a basal, electrogenic H^+ V-ATPase energizes luminal alkalization in the midgut of larval mosquitoes (Boudko et al., 2001b; Zhuang et al., 1999). Although the electrogenic transport of K^+ drives the pH gradient, there must also be flux of one or more weak anions in the opposite direction to maintain homeostasis. Several transporters are thought to participate in this mechanism.

Another line of evidence suggests that the levels of carbon dioxide in the hemolymph of lepidopterans are lower than those within the midgut lumen. The concentration of CO_2 has been determined to be near 5 mmol l^{-1} in the hemolymph and 50 mmol l^{-1} in the midgut lumen in larval *Hyalophora cecropia* (Turbeck and Foder, 1970). Recent measurements using capillary zone electrophoresis of larval *Aedes aegypti* fluids have revealed a bicarbonate/carbonate level as high as 50.8±4.21 mmol l^{-1} in the midgut lumen compared with 3.96±2.89 mmol l^{-1} in the hemolymph (means ± S.E.M., N=4) (Boudko et al., 2001a). These values correlate with those observed by Turbeck and Foder (1970). This combined evidence suggests that the CO_2 that reaches the midgut lumen in the larvae of lepidopterans is rapidly converted to a mixture of bicarbonate and carbonate. The role of carbonic anhydrase in the alkalization process would be of great significance.

Additional evidence involving the transport of CO_2 comes from studies performed on the rectal salt gland of *Aedes*

dorsalis (Strange and Phillips, 1984; Strange et al., 1984). The pronounced inhibitory effect of serosal acetazolamide suggests that carbonic anhydrase may also play a critical role in bicarbonate secretion by the salt gland. The generation of antibodies against A-CA will facilitate a detailed analysis of the cellular and subcellular distribution of this key enzyme in this system.

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References

Boudko, D. Y., Moroz, L. L., Harvey, W. R. and Linser, P. J. (2001a). Alkalinization by chloride/bicarbonate pathway. *Proc. Natl. Acad. Sci. USA* **98**, 15354–15359.

Boudko, D. Y., Moroz, L. L., Linser, P. J., Trimarchi, J. R., Smith, P. J. S. and Harvey, W. R. (2001b). *In situ* analysis of pH gradients in mosquito larvae using non-invasive, self-referencing, pH-sensitive microelectrodes. *J. Exp. Biol*. **204**, 691–699.

Clark, T. M., Koch, A. and Moffett, 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.

Clements, A. N. (1992). *The Biology of Mosquitoes*, vol. I (ed. A. N. Clements). London: Chapman & Hall. 100pp.

Darsie, R. F. and Morris, C. D. (2000). *Keys to the Adult Females and Fourth Instar Larvae of the Mosquitoes of Florida (Diptera, Culicidae).* Florida: Bulletin of the Florida Mosquito Control Association.

Donaldson, T. L. and Quinn, J. A. (1974). Kinetic constants determined from membrane transport measurements: carbonic anhydrase activity at high concentrations. *Proc. Natl. Acad. Sci. USA* **71**, 4995–4999.

Dow, J. A. (1984). Extremely high pH in biological systems: a model for carbonate transport. *Am. J. Physiol*. **24**, R633–R636.

Fernley, R. T. (1988). Non-cytoplasmic carbonic anhydrases. *Trends Biochem. Sci*. **13**, 356–359.

Gubler, D. J. (1997). Dengue and dengue hemorrhagic fever. Its history and resurgence as a global public health problem. In *Dengue and Dengue Hemorrhagic Fever* (ed. D. J. Gubler and G. Kuno), pp. 1–22. Wallingford, UK: CAB International.

Halstead, S. B. (1997). Epidemiology of dengue and dengue hemorrhagic fever. In *Dengue and Dengue Hemorrhagic Fever* (ed. D. J. Gubler and G. Kuno), pp. 23–44. Wallingford, UK: CAB International.

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

Harvey, W. R. (1992). Physiology of V-ATPases. *J. Exp. Biol.* **172**, 1–17.

Haskell, J. A., Clemons, R. D. and Harvey, W. R. (1965). Active transport by the *Cecropia* midgut. I. Inhibitors, stimulants and potassium-transport. *J.Cell. Comp. Physiol*. **65**, 45–56.

Hewett-Emmett, D. (2000). Evolution and distribution of the carbonic anhydrase gene families. *EXS* **90**, 29–76.

Jungreis, A. M., Barron, D. N. and Johnston, J. W. (1981). Comparative properties of tobacco hornworm *Manduca sexta*, carbonic anhydrases. *Am. J. Physiol.* **241**, R92–R99.

Martin, M. M., Martin, J. S., Kukor, J. J. and Merrit, R. W. (1980). The digestion of protein and carbohydrate by the stream detritivore, *Tipula abdominalis* (Diptera, Tipulidae). *Oecologia* **46**, 360–364.

Meldrum, N. U. and Roughton, F. J. W. (1933). Carbonic anhydrase: Its preparation and properties. *J. Physiol., Lond.* **80**, 113–142.

Osborne, W. R. A. and Tashian, R. E. (1975). An improved method for the purification of carbonic anhydrase isozymes by affinity chromatography. *Anal. Biochem.* **64**, 297–303.

Ridgway, R. L. and Moffett, D. F. (1986). Regional differences in the histochemical localization of carbonic anhydrase in the midgut of tobacco hornworm (*Manduca sexta*). *J. Exp. Zool*. **237**, 407–412.

Silverman, D. N. and Tu, C. K. (1986). Molecular basis of the oxygen exchange from CO_2 catalyzed by carbonic anhydrase III from bovine skeletal muscle. *Biochemistry* **25**, 8402–8408.

Spielman, A. and D'Antonio, M. (2001). *Mosquito: A Natural History of our most Persistent and Deadly Foe*. New York: Hyperion Press.

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. and Phillips, J. E. (1984). Mechanism of CO_2 transport in rectal salt gland of *Aedes*. I. Ionic requirements of CO_2 secretion. *Am. J. Physiol.* **246**, R727–R734.

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. *Am. J. Physiol.* **246**, R735–R740.

Tashian, R. E. (1969). In *Biochemical Methods in Red Cell Genetics* (ed. J. J. Yunis), pp. 307–336. New York: Academic Press Inc.

Turbeck, O. B. and Foder, B. (1970). Studies on a carbonic anhydrase from the midgut epithelium of larvae of Lepidoptera. *Biochim. Biophys. Acta* **212**, 139–149.

Volkmann, A. and Peters, W. (1989a). Investigations on the midgut caeca of mosquito larvae. I. Fine structure. *Tissue Cell* **21**, 243–251.

Volkmann, A. and Peters, W. (1989b). Investigations on the midgut caeca of mosquito larvae. II. Functional aspects. *Tissue Cell* **21**, 253–261.

Westerfield, M. (1994). *The Zebrafish Book: A Guide for the Laboratory Use of Zebrafish (*Brachydanio rerio*)*, section 9 (ed. M. Westerfield), p. 16. University of Oregon Press.

Wieczorek, H., Gruber, G., Harvey, W. R., Huss, M. and Merzendorfer, H. (1999). The plasma membrane H^+-V-ATPase from tobacco hornworm midgut. *J. Bioenerg. Biomembr*. **31**, 67–74.

Zhang, Y. and Frohman, M. A. (1997). Using rapid amplification of cDNA ends (RACE) to obtain full-length cDNAs. *Meth. 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*). *J. Exp. Biol*. **202**, 2449–2460.