

# *Mytilus galloprovincialis* carbonic anhydrase II: activity and cDNA sequence analysis

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**Abstract.** The esterase activity of carbonic anhydrase (CA) was investigated in mussels sampled at 24 locations along the Croatian coast of Adriatic Sea. The gills were the target tissue because the respiratory, ionic transport and pH regulatory enzyme function of CA and its potential usage as biomarkers of environmental pollution was the main topic. Total esterase activity was measured in cytosolic fraction by colorimetric end-point reaction using p-nitrophenyl acetate as enzyme substrate. CA activity was estimated by the same enzymatic reaction using acetazolamide as a specific CA inhibitor. The results of total esterase activities in winter (March; all sites average value  $0.137\pm 0.057$ ) were lower than determined for summer season (August;  $0.153\pm 0.036$ ) at almost all investigated locations. CA activities determined in gills of mussel sampled in winter ranged from 1.75% to 24.65% of total esterase activities and in summer samples were lower (0.83 to 13.45%). Although recent research showed potential application of CA activity in bioassay and biomarker in pollution studies, further research is needed. Here we report a short-term simple colorimetric microplate method which can be applied for analyses of large numbers of samples. Furthermore in this study we characterized full length coding sequence (cDNA) of *M. galloprovincialis* carbonic anhydrase II (CAII). The CAII cDNA (with the 5' and 3' untranslated regions) is 1317 bp long. The putative open reading frame encodes a polypeptide of 256 amino acids, with a theoretical pI/Mw 5.87/28.416 kDa and conserved domains (active site and zinc binding site).

## 1. Introduction

Carbonic anhydrase (CA, EC 4.2.1.1; AN: Carbonate hydro-lyase, Carbonic anhydrase, Carbonic dehydratase) are ubiquitous metalloenzymes, present in prokaryotes and eukaryotes. CAs catalyse the reversible dehydration/hydration reaction of carbon dioxide (CO<sub>2</sub>) using zinc as a cofactor [1,2].  $\alpha$ -CAs are present in vertebrates, bacteria, algae and plants;  $\beta$ -CAs are predominant in bacteria, algae and plants;  $\gamma$ -CAs are mainly present in Archaea and some bacteria;  $\delta$ -CAs and  $\zeta$ -CAs are only found in some marine diatoms [3].

Based on sequence information and functional domains, carbonic anhydrases in animals are referred to as  $\alpha$ -CAs and are all monomeric. To date 16 isozymes, 11 functional CAs, 3 CA-related proteins (CA-RPs), and 2 receptor-type protein tyrosine phosphatases have been reported in mammals [4]. Some other authors reported three distinct groups of CA isozymes within the  $\alpha$ -CA gene family. One of these groups contains the cytoplasmic CAs, which includes mammalian CA I, II, III, V, VII and XIII. Another group of isozymes, termed as membrane-bound CAs, consists of mammalian CA IV, IX, XII, XIV and XV. A third group includes several poorly known isozymes termed as CA-RPs and includes CA VIII, X and XI. CA-RP isozymes have lost classical CA activity and have an unknown physiological function [5].

In animals the diverse CA isozymes are present in diverse tissues and are involved in a number of different physiological processes, calcification-biomineralization (including bone resorption), ion transport, acid-base transport, and a number of different metabolic processes such as biosynthetic reactions. The  $\alpha$ -class is by far the best studied with respect to mechanism of catalysis in mammals. There are several CAs which have also shown esterase activity. Esterases are enzymes known to hydrolyse endogenous substrates, usually lipid ester substrates. *In vitro*, the esterase activity of CA was measured, both in physiological and pathological conditions [6,7,8]. Together with Na,K-ATPase, CAs represent key enzymes involved in the adaptation of marine organisms to environmental conditions [9]. Experimental studies performed on crustacean gills have shown two main isozymes of CA located in membrane-bound and in cytosolic fractions, functionally similar to mammalian CAIV and CAII [10]. The  $\alpha$ -CA isozymes differ in their kinetic properties, functions, tissue distribution and subcellular localization, and in their susceptibility to various inhibitors (11). However, little is known about the nucleotides/protein structure and *in vivo* activity of CAs in molluscs. CA activity is affected by pollution and/or natural factors such as organism metabolism, growth, reproduction, etc. [12,13]. In spite of the CA fundamental role in animal physiology only few studies are available in literature about its sensitivity to chemical pollutants. Early studies demonstrated the sensitivity of CA activity to dichloro-diphenyl-dichloroethane (DDT) exposure in birds [14], more recently several authors reported the sensitivity of this enzyme to heavy metals. Heavy metals are known to inhibit CA activity *in vitro* in a variety of organisms, including fishes [15, 16, 17], crabs [18, 19], anemones and corals [12], bovines [20], and humans [21].

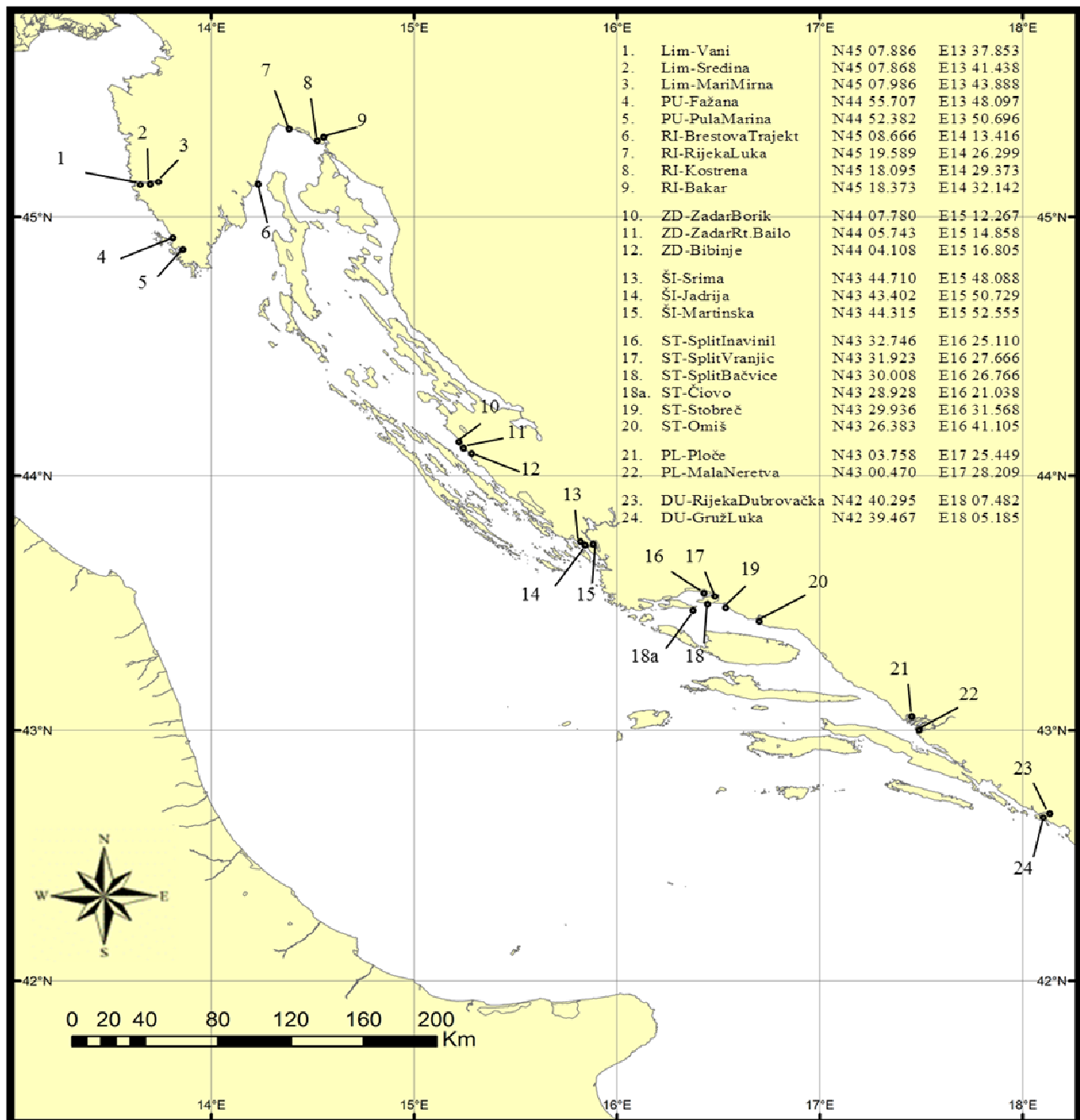
Lionetto et al. [22] investigated CA activity inhibition by heavy metals in the mussel *Mytilus galloprovincialis* in the frame of a potential application of carbonic anhydrase activity in bioassay and biomarker studies. As a filter feeding organism, *M. galloprovincialis* is known to bioaccumulate heavy metals in its tissues including shells; therefore it must cope with high concentrations of these elements [23]. Moreover, the mantle CA activity was significantly inhibited following *in vitro* and *in vivo* exposure to cadmium [8]. That research is in concordance with results that exposure to heavy metals significantly decreased shell growth reported by Soto et al. [24]. Caricato et al. [25] described tissue-specific response; heavy metal exposure triggers a decrease of CA activity in mantle but enhances CA activity and expression in digestive gland.

The use of biomarker approach in studying the effects of environmental pollutants on mussels has been reported to be very informative about the organism's stress response to toxicants [26,27]. Even though *in vitro* studies show a clear inhibition of CA activity with heavy metals [22], there are no reports about the *in vitro* esterase activity of CAs on total esterase activity level in mussel, and their possible use as biomarkers of exposure to pollutants. Therefore, in this work, carbonic anhydrase activities on basic esterase activities were measured in mussels collected along the Adriatic coast during winter and summer. In addition, the sequencing and the analysis of the most active and abundant cytosolic CAII were performed.

## 2. Materials and methods

### 2.1. Study area

In this study the esterase activity of CA on total esterase activity level was investigated in mussels *Mytilus galloprovincialis* sampled from natural populations along the Adriatic coast within the frame of Croatian National Monitoring Programme “Project Adriatic” (Fig. 1).



**Fig. 1.** Sampling locations along the Croatian coastal area of the Adriatic Sea. The codes and geographical coordinates are described on the right upper part of the map.

The Adriatic Sea is a long semi-enclosed basin of the Mediterranean. Altogether 24 locations were chosen for the study. The investigations were focused on hot spots and sensitive areas. A hot spot

was defined as a marine environment under the influence of different contamination sources that could affect human health, ecosystems, biodiversity and sustainability of the economy to a significant degree. Sensitive areas represent natural estuaries and coastal waters of socio-economic value that have a higher risk of suffering impacts from human activities. Along the eastern Adriatic coast sampling sites comprising maricultured (site No. 1), protected (site No. 2, 3), urban (site No. 8, 9, 10, 11, 13, 14, 15, 18), industrial (site No. 4, 16, 17, 20), harbour (site No. 5, 6, 7, 12, 19, 24), brackish (site No. 22, 23) and referent-pristine (site No. 21) areas were selected (Fig. 1).

## 2.2. Organism and collection

Bivalve molluscs *Mytilus* sp. have been widely used as sentinel organisms in biomonitoring programmes in order to assess the quality of marine environment. Mussels from sampling location are identified by morphological and PCR nuclear marker Me 15/16 analyses as *Mytilus galloprovincialis* Lamarck, 1819 [27]. Mussels (4.5 to 5 cm shell length), were collected below the low-high tide zone, from depth of 0.5-1 m at all sites. Temperature and salinity were measured *in situ*, at depth of mussel sampling, using a Multiline P4 universal pocket meter (WTW). Temperature values measured during the sampling in winter (10-14°C) and summer period (16-27°C) were in the range of normal oscillations typical for the eastern part of the Adriatic Sea [28]. The salinity ranged between 10 and 37 psu (practical salinity unit), depending on fresh water input and season. After sampling, mussels were dissected within 2 h of collection. Gills were excised, chilled and stored in liquid nitrogen until final storage in the laboratory at -80°C.

## 2.3. Methods

### 2.3.1. Sample preparation

Gills tissue was homogenised in cold (4°C) 0.1 M Tris HCl buffer (pH 7.3), using a small Eppendorf tube homogeniser. The gill and buffer ratio was 1:4 (w:v, 100 mg:400 µl). In all the tissue homogenates, PMSF (phenylmethylsulfonyl fluoride, 0.5 mM final concentration) was added to inhibit proteases. Tissue protein concentration was determined in homogenates using method described by Bradford (1976) with bovine serum albumin (BSA) as protein standard.

### 2.3.2. Reagents and stock solutions

Purified human erythrocyte Carbonic anhydrase II (CAII) Sigma (1 mg/ml in deH<sub>2</sub>O); 4-nitrophenyl acetate (4-NPA) Sigma 10 mM 4-NPA in 95% ethanol (dissolved in DMSO at 1 M, then diluted 100 fold); Acetazolamide (AZM) Sigma-Aldrich (1 mM AZM in 50% DMSO).

### 2.3.3. Esterase activity measurement by 4-nitrophenylacetate (4-NPA) hydrolysis assay

CA enzymatic activity was measured by a modification of the method early described by Pocker and Stone [29] on gills homogenate. In this assay, 4-NPA, which is colorless, is hydrolyzed to 4-nitrophenol and acetate and the reaction is followed by measuring the formation of 4-nitrophenol, a yellow colored compound. Measurement was performed immediately after centrifugation of the homogenate at 10000 g for 10 min. Enzyme activity was determined in duplicate in each of the 3 individuals per sampling site. The reaction was performed at room temperature (25°C) in 210 µl (total volume) using 96-well plates. In preliminary experiments, phosphate, HEPES or Tris buffered reaction media were compared and found to yield similar results. In subsequent experiments, Tris was used to buffer the reaction medium at a total concentration of 20–25 mM, and at pH 7.3. The reaction was initiated by addition of either the enzyme solution or 4-NPA solution. Immediately after the reaction was initiated, the plate was placed in a Multiscan microplate reader (Labsystems,

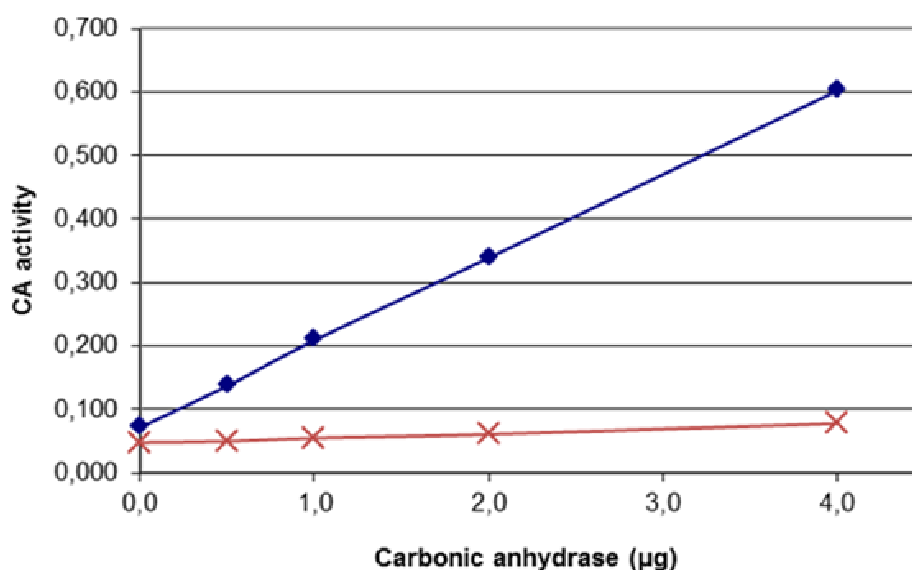
Multiscan Ascent<sup>®</sup> and Ascent Software TM, 2.4. version) and the amount of reaction product formed was monitored (1-10 min) at 405 nm.

A reference inhibition measurement was obtained by preparing the same reaction without enzyme solution (control/blank). The activity and inhibitory effect of acetazolamide to CAII was examined (Fig. 2). Range of AZM used concentration was tested in triplicate and it was proved that 0.05 mM 4-NPA inhibited CA esterase activity completely, what is in concordance with acetazolamide IC<sub>50</sub> Value for Human *Homo sapiens* isoform CAII (0.0263 mM) previously reported using 4-nitrophenyl phosphate as substrate, at pH 7.4 and 25°C [13]. In addition, the time course of CA enzymatic reaction determined in the presence of 0.5 mM AZM was statistically indistinguishable from the time course obtained in the absence of the enzyme suggesting a high sensitivity of mussel CA to sulphonamides inhibition.

For each sample, the reaction tube contained:

- 10 µl Sample (10 µg protein) or CA standard (0.1-4.0 µg)
- 125 µl 50 mM Tris
- 65 µl 10 mM 4-NPA substrate dissolved in 95% ethanol
- 10 µl 1 mM AZM in 50% DMSO (+AZM, CA inhibition assay)  
or 50% DMSO (-AZM, Total esterase assay)

The specific activity can be expressed in nmoles of hydrolysed p-NPA per min per mg of protein (nmol/min/mg); molar extinction coefficients at 405 nm ( $18.5 \text{ mM}^{-1}\text{cm}^{-1}$ ). One unit of activity represents the amount of enzyme catalysing 1 nmol reaction per min under the assay conditions. In our case we did not know esterase (carbonic anhydrase) protein content in our gill extract samples, therefore only hydrolysis activity was measured at the same amount of total protein content (10 µg). Using specific detection with 5-Dimethylaminonaphthalene-1-Sulfonamide (DNSA) fluorescent CA inhibitor and SDS PAGE, it was possible to separate and visualise weak band at about 30 kDa, but for our purposes, preliminary research, and just to compare total esterase activities with CA inhibited activities between locations it was not necessary to determine specific activity.



**Fig. 2.** Acetazolamide inhibition and esterase activity measurement of CAII enzyme standards (red crosses and blue diamonds-lines, respectively).

#### 2.3.4. Data analysis

Results are presented as mean values  $\pm$  standard deviations (SD). The correlation analyses were carried out using the STATISTICA software package (StatSoft, USA).

#### 2.3.5. cDNA preparation

Mediterranean mussels *M. galloprovincialis* were obtained from mariculture in Limski kanal, Adriatic Sea, Croatia. Mussel gills tissue (15 mg) of one specimen was snap-frozen and ground in liquid nitrogen, and further total RNA was isolated using RNeasy Protect Kit (Qiagen) according to manufacturer instructions. The RNA was eluted in 40  $\mu$ L of elution buffer. Total RNA (10  $\mu$ L; estimated to 1  $\mu$ g) was transcribed into cDNA in a reaction volume of 50  $\mu$ L using High Capacity cDNA Archive Kit and protocol (Applied Biosystems).

#### 2.3.6. Cloning and sequencing of *M. galloprovincialis* CA cDNA

*M. galloprovincialis* CA cDNA was amplified by polymerase chain reaction (PCR). Degenerated primers pairs were designed according to conserved regions (QSPINI and GSLTTP protein regions) of a few known CA sequences of sponges, molluscs and vertebrates. Primers F\_CA\_QSPIDI and R\_CA\_GSLTTP were used to amplify our first partial CA sequence, followed by cloning and sequencing in gel (Licor Odyssey). This sequence was compared and analysed for similarity to other known sequences in public-domain databases (e.g. the NCBI GeneBank, [www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)). This part of work was done in 2008 as a side result of characterization of biomineralization enzymes (e.g. nacrein) of freshwater and marine mussels in the frame of Croatian-German bilateral cooperation project (MOE 08/R55). Further work to obtain full CAII cDNA sequence was done later, using *M. galloprovincialis* cDNA Library. The cDNA library was done by Macrogen service with following Construction Report: A) Source of RNA - Total RNA conc. 625.97 ng/ $\mu$ L, A260/A280 = 2.13, A260/A230 = 2.31, B) Library - Excised Phagemid Type, Vector pBluescript® SK-, Insert ratio >100, Insert size distribution 0.5 – 3 kb, Titer 1.0 x 10<sup>6</sup> cfu/ml, Host strain SOLR strain).

The applied PCR and sequencing strategy was as follows: Middle part - Primers F\_CA\_QSPIDI and R\_CA\_GSLTTP were used to amplify clone 1; Primers CA2\_F1 and T3 for clone 2; Primers CA2\_R1 and T7 for clone 3 cDNA sequences (Table 1). The final sequence was assembled as a contig.

**Table 1.** Primer sequences.

Names	Sequences (5'-3')
T7	TAATACGACTCACTATAGGG
T3	AATTAACCCTCACTAAAGGG
F_CA_QSPIDI	TCGCCGCAATCGATAHDB
R_CA_GSLTTP	GTGGCGTTGTCAGTGAACC
CA2_F1	AGGTTCTGAACACACAATCG
CA2_R1	GACTGGCCATGTCTAAATCA

PCR reactions contained 1.25 U of Taq DNA Polymerase (Fermentas) in 1 $\times$  Taq Buffer with KCl, 0.2 mM dNTPs (Fermentas), 2 mM MgCl<sub>2</sub>, 2  $\mu$ L cDNA or cDNA Library and 0.3  $\mu$ M primers in a reaction volume of 50  $\mu$ L. Thermal cycling conditions were as follows: initial denaturation 2 min at

95°C; 30 cycles (30 s at 95°C, 30 s at 52/56/58°C, 1 min at 72°C); final extension 7 min at 72°C; sample storage at +4°C until gel electrophoresis.

PCR products of expected sizes were excised from a preparative agarose gel and purified using High Pure PCR Product Purification Kit and protocol (Roche, Mannheim, Germany). Purified PCR products were cloned using TOPO TA Cloning Kit and protocol (Invitrogen Life Technologies). Plasmid DNA was isolated by High Pure Plasmid Isolation Kit (Roche). All sequencing was performed through the sequencing service of Macrogen Inc. (Republic of Korea).

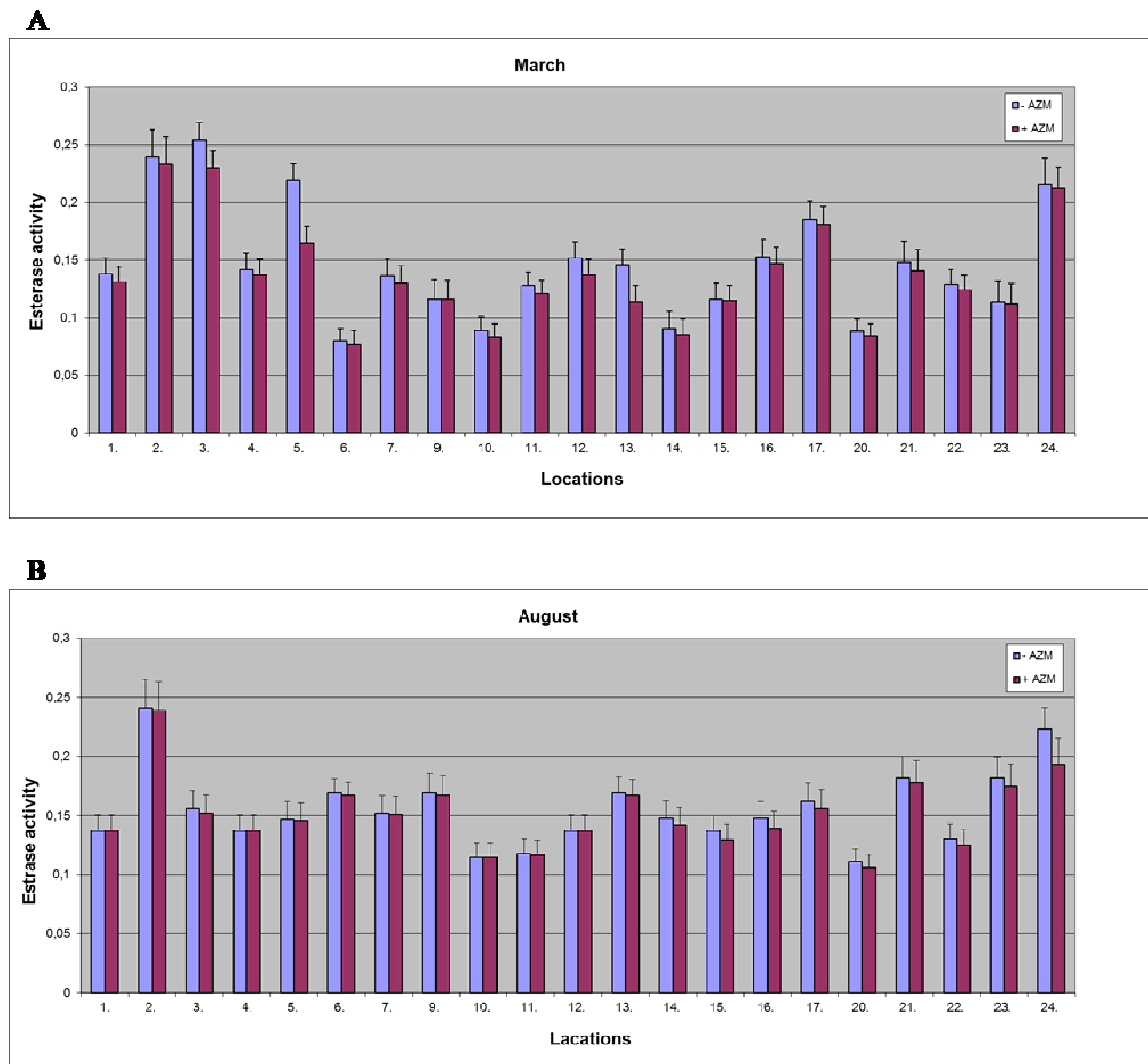
#### 2.3.7. Bioinformatic and Phylogenetic analysis

The NCBI GenBank nucleotide sequence database was screened for CAII sequences from other organisms. The sequences were analysed using Lasergene 7 (DNASTAR Inc. Madison WI) program package and ClustalX software. For the functional annotation of proteins, carbonic anhydrase search of Conserved Domain Database (NCBI) was done. A molecular phylogenetic tree was constructed with the use of a Neighbor-Joining (NJ) method by Geneious<sup>®</sup> software ver. 7.1.7 (Biomatters Ltd).



### 3. Results

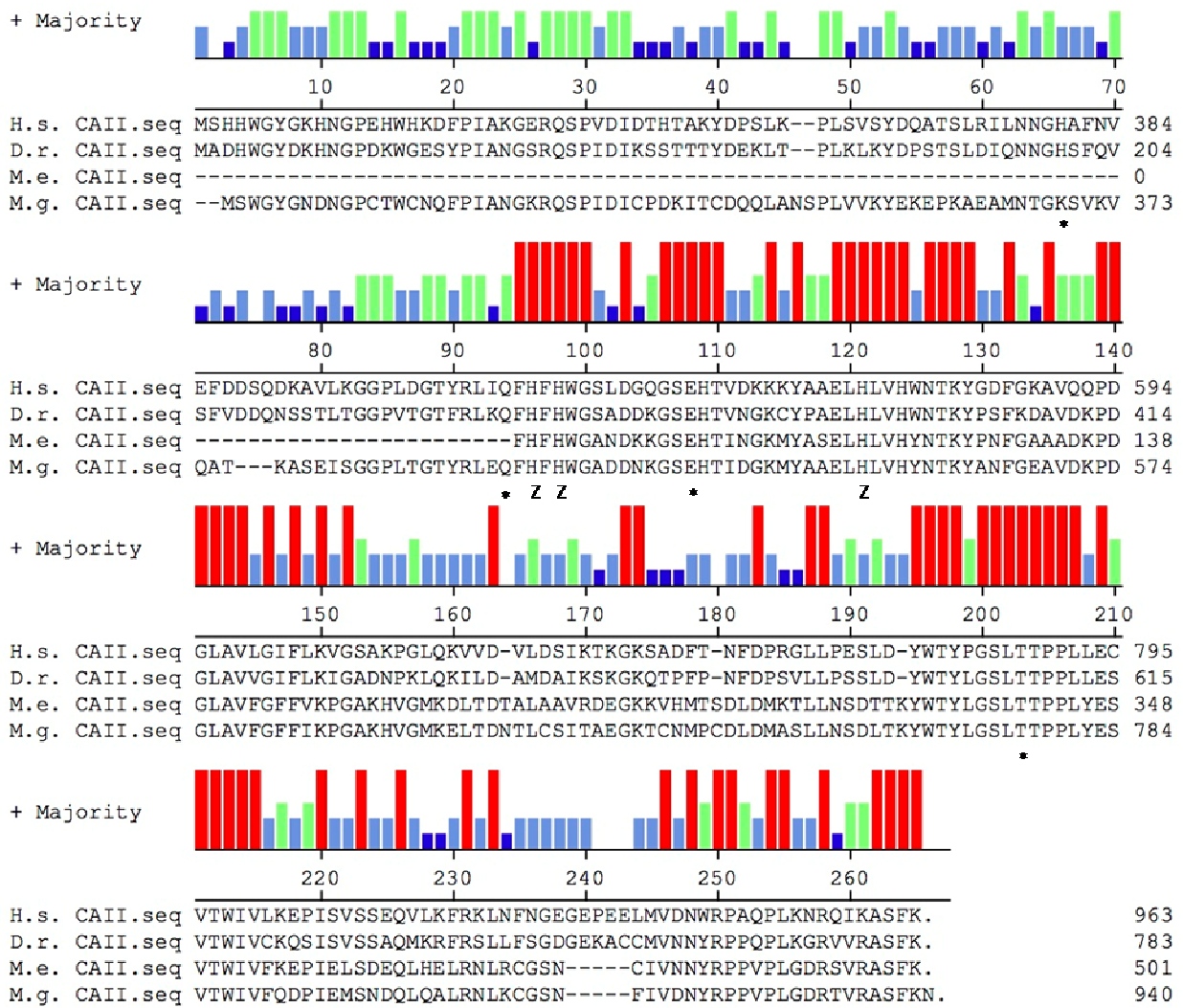
The results of total esterase activities in gills of mussels in winter (average value March;  $0.137 \pm 0.05$ ) were lower than those determined for the summer season (August;  $0.153 \pm 0.036$ ) at almost all investigated locations (Fig. 3). CA activities determined in gills of mussels sampled in winter ranged from 1.75% to 24.65% (average  $6.21 \pm 6.38$ ) of total esterase activity. CA activities in summer samples were from 0.83 to 13.45% ( $2.83 \pm 3.16$ ).



**Fig. 3.** Total esterase activity in cytosolic fraction of mussel gills: A) Winter and B) Summer period; (-AZM) without specific CA inhibitor and (+AZM) with acetazolamide inhibition.

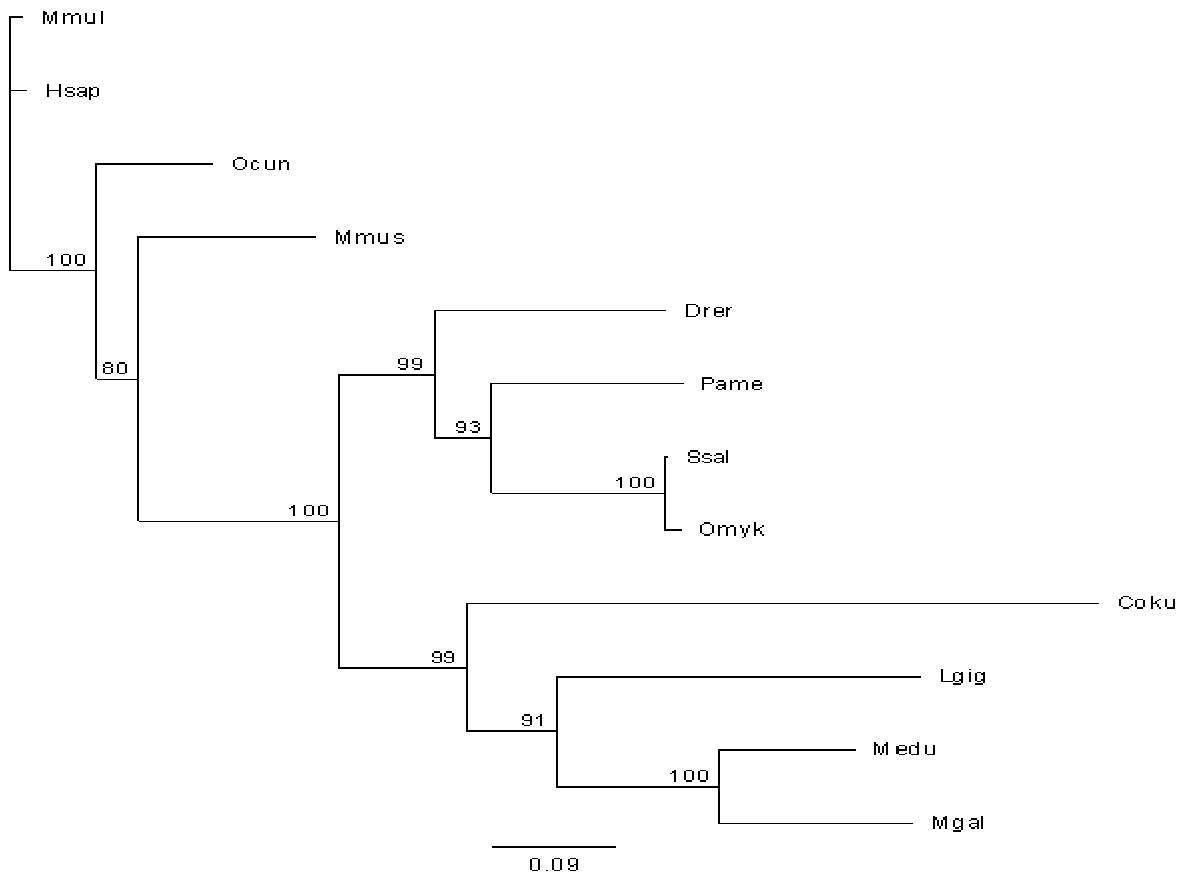
The full-length sequence of *M. galloprovincialis* CAII was obtained from gills cDNA library. The CAII cDNA transcript with the 5' and 3' untranslated regions is 1317 bp long (Fig. 4). The putative open reading frame (170-940 bp) encodes a polypeptide of 256 amino acids, with a theoretical pI/Mw 5.87/28.416 kDa and conserved domains, corresponding to the active site and zinc binding site. According to the NCBI database, in molluscs, CA sequence data including Nacrein and Nacrein-like proteins are relatively abundant, but are focused on a limited number of species.





**Fig. 5.** Alignment of our *M. galloprovincialis* cDNA-translated sequence with similar human *Homo sapiens* (NP\_000058.1), fish *Danio rerio* (NP\_954685.1) and partial mussel *Mytilus edulis* (CDU21917.1) CAs retrieved after NCBI blast. The homologous conserved regions are shaded with green and red bars. The histidine residues involved in zinc binding in the catalytic site are indicated by a Z, and \* signs represented amino acids participating in the hydrogen bond network forming the active site.

From BLAST results and the alignment shown in Figure 5 and 6, the putative CAII amino acid sequence from *M. galloprovincialis* exhibits strong similarities with the other  $\alpha$ -CAII sequences. The three histidine residues (shown by a Z) directly involved in binding the zinc cofactor are conserved in our sequence. The amino acids involved in the associated hydrogen bond network surrounding the active site (indicated with asterisks \* signs) are also highly conserved.



**Fig. 6.** Unrooted neighbor-joining (NJ) tree obtained with multiple alignment of 12 CAII amino acid sequences. Accession numbers of the protein sequences used as a matrix are as follows (NCBI): Medu - *Mytilus edulis* (CDU21917.1), Lgig - *Lottia gigantea* (XP\_009052992.1), Pedu - *Pseudopleuronectes americanus* (AAV97962.1), Ssal - *Salmo salar* (NP\_001133769.1), Drer - *Danio rerio* (NP\_954685.1), Omyk - *Oncorhynchus mykiss* (NP\_001117693.1), Coku - *Calyptogena okutanii* (BAO01208.1), Mmul - *Macaca mulatta* (NP\_001182346.1), Mmus - *Mus musculus domesticus* (AAA37357.1), Hsap - *Homo sapiens* (NP\_000058.1) and Ocun - *Oryctolagus cuniculus* (NP\_001182637.1).

The *M. galloprovincialis* CAII shows homology with other molluscan and also vertebrate carbonic anhydrases (Fig. 5 and 6), especially in the highly conserved QSPIDI and GSLTTPP regions. According to our analysis CA is likely to be a homolog of  $\alpha$ -CAII [30].

#### 4. Discussion

In contrast to the chemical measurement of contaminants accumulated in body tissues, biomarkers can give a more complete and relevant information on the impact of toxic compounds on the health of organisms [26]. At present many authors report multi-biomarker studies in wild animals collected from the field or in transplanted animals. *Mytilus* sp. is widely used as sentinel organisms in marine environmental monitoring and assessment programs. The possible application of CA activity inhibition as an *in vitro* bioassay for the detection of heavy metals in pollution monitoring using the mussel *M. galloprovincialis* has previously been suggested [12, 22].

The investigated sites along the Adriatic coast are located in the vicinity of the urban areas, Pula, Rijeka, Zadar, Šibenik, Split and Dubrovnik. These are coastal areas under the influence of human activities. In general PAH concentrations in seawater vary widely from lower than the detection limit up to 305 ng/l and are in the range similar to those reported for the Thermaikos Gulf, Greece. Further the levels of PCBs in the Adriatic sediment samples analysed during the 1976-1992 period (60 sampling sites) were significantly lower than in samples from areas of the south coast of France, the west coast of Italy and the east coast of Greece [31].

Seasonal changes of heavy metal pollution in the soft tissue of *M. galloprovincialis* from the middle Adriatic were observed previously [32]. The concentrations of Pb ranged from 2 to 7 ppm, As from 4 to 30 ppm, Cr from 1 to 2.9 ppm, Mn from 2 to 13 ppm, Fe from 53.4 to 719 ppm, Ni from 0.8 to 5 ppm, Cu from 3.7 to 11.1 ppm and Zn from 59.1 to 273 ppm. Maximum concentrations were measured from the heavily polluted Vranjic region (Site No. 17). In our previous study trace metal concentrations for *M. galloprovincialis* shells from Vranjic were 1.79 ppm for Cu, 2.29 ppm for Pb, 0.90 ppm for Zn, 6.0 ppm for Ni, 7.00 ppm for Mn, 1 ppm for Cr, 0.2 ppm for As and <0.02 ppm for Fe [23].

According to the trace element chemistry of the *M. galloprovincialis* shells, the most polluted sample locations were in the middle Adriatic also, where chemical and heavy industries are located and where sewage is discharged into the coastal sea. The highest concentrations of zinc, lead and copper were measured in samples from Pula (No. 5), Rijeka (No. 6) and Gruz (No. 24) where there are also ports in addition to industry. According to measured elements two groups were identified: 12 more polluted locations and 10 less polluted locations [23].

Both environmental and physiological factors appear to have an influence on the incorporation of trace elements into the shell. While salinity is relatively constant in the open sea, with typical values in the northern and southern Adriatic of  $37.65 \pm 0.40$  and  $38.37 \pm 0.19$  psu respectively, it varies in intertidal zones, estuaries, close to under-sea freshwater springs and during rainy days in closed lagoons (4–37 psu). At the same time, seawater temperatures exhibit spatial intra-seasonal differences in addition to strong seasonal variation. According to the measured variables of seawater temperature, salinity and  $\delta^{18}\text{O}$  and  $\delta^{13}\text{C}$  isotopes, for the Adriatic Sea biomonitoring purposes we determined locations with strong to medium freshwater influx that could possibly interfere with pollution effects in mussels: Rijeka Dubrovačka (No. 23), Rijeka (No. 7), Bakar (No. 9), Martinska (No. 15), Omiš (No. 20), Ploce (No. 21), Sv. Ivan/Mala Neretva (No. 22), Lim-inside (No. 3) and Inavinil (No. 16) [28].

The gills were the target tissue because the respiratory, ionic transport and pH regulatory enzyme function of CA and its potential usage as a biomarker of environmental pollution was the main topic. In most samples, the differences of product between the uninhibited CA-catalyzed reaction (Total Esterase Activity) and the AZM (CA-inhibited) reaction was low (2-10%) but at some sites rise up to 20-25%. Esterase activity measured as hydrolysis of 4-nitrophenyl acetate was performed

probably by different reactions catalysed by enzymes such as carboxylesterase (3.1.1.1), arylesterase (3.1.1.2), acetylsalicylate deacetylase (3.1.1.55), feruloyl esterase (3.1.1.73), palmitoyl-CoA hydrolase (3.1.2.2), acyl-CoA hydrolase (3.1.2.20), including our enzyme of interest carbonate dehydratase (4.2.1.1).

When using mussels as sentinel organisms for biomarker studies, much importance is given to oscillations of abiotic variables, particularly temperature and salinity, both commonly related to Esterase and Carbonic anhydrase activity variation. The correlation of total esterase activities (+AZM) and CA (-AZM) activities are low (March 0.269; August 0.315). Further in winter positive correlation of esterase activities with temperature (0.232) and salinity (0.344) was established in opposite to summer where the correlation was negative (temperature -0.030; salinity -0.133).

In winter CA activities (+AZM) were correlated with salinity (March 0.405; August -0.032) and in summer negatively correlated with temperature (March -0.026; August -0.383). CA activities decreased with rising of seawater temperature and total esterase activities increased. However, mussels' lower (inhibited) CA activities expressed in summer, indicating beside temperature effect, the possible effects of pollution. The level of pollution is connected with the touristic season, but effects on mussel are also depending on internal factors, in particular nutritive stress and the dynamics of reproductive cycle [26, 28].

The highest concentrations of zinc, lead and copper were measured in samples from Pula (No. 5), Rijeka (No. 6) and Gruž (No. 24) where there are also ports in addition to industry. The interpretation of elevated concentration measured in shells is related to anthropogenic pollution at locations where heavy industry is situated. Mussels give a complete record of contaminants within their lifespan, and concentrations of pollutants fluctuate in the shell to a lesser degree than in the water they live in and remain in the shell permanently.

The total esterase activities were above average values for the winter season, and CA activities at Pula (24.66), higher and Rijeka (4.41), Vranjic (2.16) and Gruž (1.85) are lower than average season value (6.20). Here we can suppose a possible inhibition of CA, under the assumption that the cumulative effect of heavy metal pollution does not necessarily have to be additive. Again, the total esterase activities are above average values for the summer season, CA activities at Pula (1.18), and Rijeka (1.18) were below, in opposite Vranjic (3.70) and Gruž (13.45) were lower than average season value (2.83).

This research constitutes a pilot project, which shows that it is difficult to assume a direct relationship between pollution at the investigated sites with the CA activities we determined. The present paper does not provide conclusive information about the chemical specificity responsible for the CA inhibition; therefore, future studies will be addressed to evaluate whether the observed response is specific for heavy metal exposure and to clarify potential biomarker application. Besides emphasizing CA as a putative biomarker of pollution, our study allowed characterizing the primary structure of CA from the edible mussel. The first discovered CA isoforms were  $\alpha$ -CAs from human red blood cells. However, in the last decades, a remarkable number of  $\alpha$ -CAs have been described from non-mammalian organisms, but for other metazoans there is still a lot of work to be done. In molluscs, sequence data is relatively abundant, but are focused on a limited number of species. According to Interpro, 120 molluscan proteins annotated with an  $\alpha$ -carbonic anhydrase domain (IPR001148) are currently deposited in the UniprotKB protein sequence database. Out of these, 72 pertain to bivalves, including several redundant entries. However, large whole-genome and whole-transcriptome highlighted that the number of CA genes is relatively large in bivalve species. Indeed, at least 34 CA-related genes (including highly similar paralogs and, possibly, pseudogenes) are

present in the genome of the oyster *Crassostrea gigas* [33]. Based on this indication, additional studies will be required to explore the diversity of functionality of molluscan CA genes. Within the deuterostomes, most of the available  $\alpha$ -CA data in echinoderms are related to the genome sequencing of the sea urchin *Strongylocentrotus purpuratus* [34].

In this paper we focused on total esterase activity and cytosolic CA activities, describing one most active CAII of common bioindicator species *M. galloprovincialis*. Our preliminary analyses of *Mytilus* CA transcriptome sequences proved the presence of different isoforms, and their cellular localization (cytosol/membrane), and until now the absence of expressed Nacrein and Nacrein-like proteins [35,36]. Because of the wide application of *M. galloprovincialis* in environmental quality monitoring and assessment, the obtained results represent a solid basis for future potential biomarker application of the CA activity in the sentinel organism *M. galloprovincialis*.

#### 4. Conclusion

Although *in vitro* studies show clear inhibition of CA activity with heavy metals, further detailed research is needed for the application of CA as a biomarker of pollution. The effects of environmental and physiological factors and different CA isozymes activity using different inhibitors must be considered. Detailed protein and gene characterizations are of great importance for the application of CA as a biomarker. In this paper we report a full length amino acid sequence of *M. galloprovincialis* CAII. According to the deduced amino acid sequence alignment, this putative *M. galloprovincialis* CA exhibits all features required for CA activity.

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