

Carbonic Anhydrase in the Scleractinian Coral *Stylophora pistillata*

CHARACTERIZATION, LOCALIZATION, AND ROLE IN BIOMINERALIZATION*

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Carbonic anhydrases (CA) play an important role in biomineralization from invertebrates to vertebrates. Previous experiments have investigated the role of CA in coral calcification, mainly by pharmacological approaches. This study reports the molecular cloning, sequencing, and immunolocalization of a CA isolated from the scleractinian coral *Stylophora pistillata*, named STPCA. Results show that STPCA is a secreted form of α -CA, which possesses a CA catalytic function, similar to the secreted human CAVI. We localized this enzyme at the calicoblastic ectoderm level, which is responsible for the precipitation of the skeleton. This localization supports the role of STPCA in the calcification process. In symbiotic scleractinian corals, calcification is stimulated by light, a phenomenon called “light-enhanced calcification” (LEC). The mechanism by which symbiont photosynthesis stimulates calcification is still enigmatic. We tested the hypothesis that coral genes are differentially expressed under light and dark conditions. By real-time PCR, we investigated the differential expression of STPCA to determine its role in the LEC phenomenon. Results show that the STPCA gene is expressed 2-fold more during the dark than the light. We suggest that in the dark, up-regulation of the STPCA gene represents a mechanism to cope with night acidosis.

Carbonic anhydrases (CA)⁴ are ubiquitous metalloenzymes that catalyze the reversible hydration of carbon dioxide into bicarbonate: $\text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{HCO}_3^- + \text{H}^+$. There are at least

five classes of CA with polyphyletic origin (1–6): α (vertebrates, invertebrates, bacteria, and some chlorophytes), β (eubacteria and chlorophytes), γ (archaea and some eubacteria), and δ and ζ (marine diatoms). Among numerous roles in physiological processes, carbonic anhydrases play an important role in biomineralization from invertebrates to vertebrates, even if this role is not well understood (7–11).

Scleractinian corals precipitate an aragonitic calcium carbonate (CaCO_3) skeleton resulting from the precipitation of calcium and inorganic carbon at the site of calcification. Several studies have demonstrated the involvement of CA in coral calcification (7, 12–18): CA could help in supplying dissolved inorganic carbon (DIC) or in removing carbonic acid from the site of skeletogenesis.

One specific feature of calcification in symbiotic corals is that calcium carbonate deposition in skeleton is higher in light than in dark conditions. This phenomenon, called “light-enhanced calcification” (LEC), is due to the symbiosis that corals establish with their photosynthetic Dinoflagellates. Despite numerous studies performed on this symbiotic relationship, the mechanisms linking photosynthesis of the symbionts to coral calcification remain largely unknown (see review in Refs. 19, 20).

In this study, we have cloned, sequenced, and localized an α -CA from the coral *Stylophora pistillata* and then determined the differential gene expression between light and dark to generate more information on the function of CA in coral calcification and in the light-enhanced calcification phenomenon. Our results allow us to propose a model to explain the role of CA in the coral calcification process.

EXPERIMENTAL PROCEDURES

Biological Materials—Experiments were conducted in the laboratory on the branching symbiotic scleractinian coral *S. pistillata* (Esper, 1797). Colonies were stored in a 300-liter aquarium, supplied with seawater from the Mediterranean Sea (exchange rate $2\% \text{ h}^{-1}$) under controlled conditions: semi-open circuit, temperature of 26.0 ± 0.2 °C, salinity of 38.2 psu, light of $175 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ (using the fluorescent tube Custom Sea Life®) on a 12:12 photoperiod. Lighting-on occurred at 9:00 AM. Corals were fed three times weekly with a mix of *Artemia salina* nauplii, *A. salina* frozen adults, and frozen krill.

RNA Extraction—Extractions of RNA were performed at 12:00 AM for the light condition and at 12:00 PM for the dark condition, because we have previously shown that calcification rates were stable in the light and dark with a 2.6-fold enhance-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) EU159467.

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⁴ The abbreviations used are: CA, carbonic anhydrase; STPCA, carbonic anhydrase from *S. pistillata*; anti-STPCA, specific antibody against the STPCA; DIC, dissolved inorganic carbon; RACE, rapid amplification of cDNA ends; ML, maximum likelihood; DMEM, Dulbecco’s modified Eagle’s medium; GPI, glycosylphosphatidylinositol; NTA, nitrilotriacetic acid; MCMCMC, Metropolis-coupled Markov Chain Monte Carlo; LEC, light-enhanced calcification; AA, amino acids.

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ment of calcification rates in light (21). Total RNAs were extracted with Trizol[®] reagent (Invitrogen) according to the Chomczynski method (22), which was slightly modified (23). Briefly, five grams of coral apices were ground into powder in a cryogrinder (Freezer/Mill 6770, Spex Sample Prep[®]) and dissolved in 20 ml of Trizol[®] reagent. Three centrifugations (10 min, 15,000 × *g*, 4 °C) were performed. 4 ml of chloroform were added to the last supernatant and incubated 2–3 min at room temperature. A centrifugation (15 min, 12,000 × *g*, 4 °C) allowed separation of two phases, an aqueous phase and a phenol-chloroform phase. The aqueous phase, containing RNA, was subjected to a second chloroform extraction (v/v). 10 ml of isopropyl alcohol were added to the second aqueous phase, vigorously shaken, and incubated for 10 min at room temperature. A centrifugation (15 min, 12,000 × *g*, 4 °C) permitted the pelleting of the RNA. This pellet was washed in 5 ml of ethanol (70%), centrifuged (5 min, 7500 × *g*, 4 °C), dried 10 min at room temperature, and then resuspended in 100–200 μl of RNase-free water (Eppendorf, Hamburg, Germany). The RNA sample was finally centrifuged (20,000 × *g*, 10 min, 4 °C) to remove the last contaminants. DNase treatment of samples was performed to avoid genomic DNA contamination (Turbo DNA-free[™] kit, Ambion). For real-time PCR experiments, mRNA from each sample was purified using the mRNA isolation kit (Roche Applied Sciences) according to the instructions of the supplier. Moreover, we performed control PCR reactions with non-reverse-transcribed samples and achieved negative results to confirm the absence of DNA contamination (results not shown).

cDNA Synthesis, PCR Amplification, Cloning, and Sequencing—Reverse transcription was performed using 50 units μl⁻¹ RTIII Superscript (Invitrogen) according to the instructions of the supplier on 2 μg of DNase-treated (Turbo DNA-free[™] kit) RNA with an oligo(dT) primer for the cloning of carbonic anhydrase and on 200 ng of mRNA using random hexamer primers for real-time PCR experiments.

PCR amplification was carried out using Platinum TaqDNA polymerase (Invitrogen) and degenerate primers: forward (5'-CAGTTNCAYTTYCAITGGGG-3') designed for the conserved Q(L/F)HFHWG region, and reverse (5'-ACCRASCACWGCCARGCC-3') designed for the conserved PDGLAVLG region. The PCR amplification product was ligated into a plasmid vector (pGEM-T easy vector system II, Promega). Competent cells *Escherichia coli* were transformed and plated on LB/ampicillin plates spread with *x*-gal and isopropyl-1-thio-β-D-galactopyranoside. White colonies were subcultured and screened for the presence of the correct insert. DNA sequencing of three independent clones was carried out on both strands with SP6 and T7 primer sequences by Macrogen, Inc. To obtain the 5'- and 3'-ends of the sequence, rapid amplification of cDNA ends (RACE) experiments were performed using the primers SP2 (5'-TGAGCTCTTCCTTGGTAAACAC-3') and SP3 (5'-AAATTTTCTTCCACCGACCTG-3') for 5'-RACE and SP4 (5'-GCGGTTTCAGAGCATCAGGTC-3') and SP5 (5'-TGTACTIONGCAATGCGGAAAAG-3') for 3'-RACE. Both ends were cloned in pGEM-T easy vector and sequenced as mentioned above.

Sequence and Phylogenetic Analyses—BLASTX analyses were conducted on the NCBI server in the GenBank[™] data

base. The signal peptide presence was determined using the server SignalP-3 (24). Phosphorylation and glycosylation prediction analyses were performed on the Centre for Biological Sequence analysis prediction server (25). GPI (glycosylphosphatidylinositol) modification site prediction was performed using both the big-PI predictor (26) and DGPI servers, and the presence of the transmembrane domain was performed using the Tmpred (27) and NPS servers.

The alignments of carbonic anhydrase amino acid sequences were performed with ClustalW in MEGA 3.1. A phylogenetic tree was constructed with both the Maximum Likelihood (ML) and Bayesian methods. The best-fitting model of protein sequence evolution was selected using ProtTest 1.3 (28) among a set of 53 candidate models constituted through all combinations of the empirical amino acid substitution matrices (JTT, mtREV, mtMam, mtArt, Dayhoff, WAG, rtREV, cpREV, Blosum62, and VT) with a gamma distribution with four rate categories, a proportion of invariable sites (+I), and observed amino acid frequencies (+F). All statistical criteria selected WAG+G+F as the best-fitting model, with WAG+I+G +F coming a close second. To check for concordance of the results, Phyml with either a BIONJ (29) or a Bayesian (30) defined starting tree were used to obtain ML trees under a concatenated model assuming the WAG amino acid substitution matrix model and the parameter values previously estimated by ProtTest. The ML bootstrap proportion was obtained after 100 (MrBAYES and BIONJ) bootstrap replicates. Bayesian inferences were performed with MrBayes v3.1.2. Each Bayesian analysis used 4 Metropolis-coupled Markov Chain Monte Carlo (MCMCMC). Bayesian posterior probabilities were obtained from the majority rule consensus of the tree sampled after the initial burn-in period, as determined by checking the convergence of likelihood values across MCMCMC generations. 1,000,000 MCMCMC generations with sampling every 100 generations were run under the WAG+G+F model.

Synthesis of a Specific Antibody—Synthesis of peptide antigen and production of antiserum were performed by Eurogentec. The peptide (H₂N-RDP EGP DTW KHH YKD C-COOH) corresponds to the N-terminal sequence of the protein with the peptide signal removed. Briefly, the peptide with Freund's complete adjuvant and coupled to KLH (keyhole limpet hemocyanin) was injected intraperitoneally into two rabbits to elicit an immune response. Prior to injection, a blood sample from each rabbit was collected to serve as control preimmune serum. Every 2 weeks, rabbits were boosted with the same amount of antigen. Three months after the initial injections, the final bleeds containing the specific antibody against the carbonic anhydrase from *S. pistillata*, anti-STPCA, were collected, divided into aliquots, and stored at -20 °C for further immunological analyses.

Immunolocalization with Anti-STPCA—Apexes of colonies were prepared for immunolocalization as described previously by Puvarel *et al.* (31). Briefly, apices of *S. pistillata* were fixed in 3% paraformaldehyde in S22 buffer (450 mmol liter⁻¹ NaCl, 10 mmol liter⁻¹ KCl, 58 mmol liter⁻¹ MgCl₂, 10 mmol liter⁻¹ CaCl₂, 100 mmol liter⁻¹ Hepes, pH 7.8) at 4 °C overnight and then decalcified using 0.5 mol liter⁻¹ EDTA in Ca-free S22 at 4 °C. They were then dehydrated in an ethanol series and

embedded in Paraplast. Cross-sections (7- μm thick) were cut and mounted on silane-coated glass slides.

Immunolocalization was performed according to the protocol previously described by Puverel *et al.* (31). Briefly, deparaffinized sections of tissues were incubated for 1 h in saturating medium (1% bovine serum albumin, 0.2% teleostean gelatin, 0.05% Tween 20 in 0.05 mol liter⁻¹ phosphate-buffered saline, pH 7.4) at room temperature. The samples were then incubated with the anti-STPCA or the preimmune serum as primary antibodies (1:200 dilution, 1 h at room temperature and overnight at 4 °C in moist chambers). After rinsing in saturating medium, samples were incubated with biotinylated anti-rabbit antibodies (Amersham Biosciences 1:250 dilution, 1 h at room temperature) as secondary antibodies. After rinsing with phosphate-buffered saline, pH 7.4, samples were finally stained for 20 min with streptavidin AlexaFluor 568 (Molecular Probes, 1:50 dilution) and 4',6-diamidino-2-phenylindole, DAPI (Sigma, 2 μg ml⁻¹). Samples were embedded in Pro-Long antifade medium (Molecular Probes) and observed with a confocal laser-scanning microscope (Leica, TCS4D).

Expression of STPCA in HEK 293 Cells—PCR products were prepared from oligo(dT) RT coral cDNAs and cloned into pIRES-DSRED mammalian expression vector (Clontech, Inc). The PCR reaction was carried out with the following primers: forward: (5'-CTAGCTAGCTAGACCATGAAACTCTCCCTATT-3') and reverse (5'-TCCCCCGGGGATCAGTGATGGTGATGGTGATGCGATCCTCTTTGGAAGCTTCGGATGACAG-3'). The recombinant vector STPCAORFHIS contains a chimeric sequence encoding the open reading frame of the STPCA fused to a polyhistidine region at the C terminus. After sequencing, the plasmid clone was introduced into HEK 293 cells with the Lipofectamine transfection reagent (Invitrogen). A positive clone (HEK-STPCA), obtained by limiting dilution, was used to produce STPCA.

Purification of Recombinant STPCA—When the HEK-STPCA cells were 70–80% confluent, the supplemented DMEM was replaced with serum-free DMEM; medium was harvested 7 days later. Then, the supernatant of culture medium was loaded onto a Ni-NTA-agarose column, to which the His₆ tag of the recombinant protein would bind. The Ni-NTA-agarose column was washed with wash buffer (20 mM imidazole, 300 mM NaCl, 50 mM NaH₂PO₄, and 0.05% Tween 20, pH 8.0), and the His₆-tagged protein was eluted with the elution buffer (200 mM imidazole, 300 mM NaCl, 50 mM NaH₂PO₄, and 0.05% Tween 20, pH 8.0). The removal of imidazole was accomplished by concentration of eluted protein, then diluting the retentate with phosphate-buffered saline using a Centricon Plus Centrifugal Filter unit (5,000 MW cut-off). The process was repeated four times. At the end of the process, the retentate was collected, and protein concentration was measured using a Qubit kit assay (Invitrogen).

Deglycosylation Experiment and Western Blotting—Recombinant STPCA was incubated overnight at 37 °C in 250 mM Tris, pH 8.0, 50 mM 1,10-phenanthroline, 0.5% Nonidet P40, 0.25% SDS, 50 mM 2-mercaptoethanol, with or without *N*-glycosidase F (Roche Applied Science). Then, proteins were subjected to 5–12% SDS-PAGE and electroblotted onto a polyvinylidene difluoride membrane. To verify protein expression, blots were probed

with the monoclonal mouse anti-histidine tag (Serotec, 1:1000 dilution) or anti-STPCA (1:3000 dilution) using the ECL Western blotting kit (Amersham Biosciences). For the anti-phosphorylation Western blot, anti-phosphoserine, anti-phosphothreonine, and anti-phosphotyrosine were supplied by Abcam®.

CA Activity and Inhibition Assay—An Applied Photophysics stopped-flow instrument was used for assaying the CA-catalyzed CO₂ hydration activity (32). Phenol red (at a concentration of 0.2 mM) was used as an indicator, working at the maximal absorbance of 557 nm, with 10 mM Hepes (pH 7.5) or Tris, pH 8.3 as buffer, and 20 mM Na₂SO₄ or 20 mM NaCl (for maintaining constant ionic strength), following the CA-catalyzed CO₂ hydration reaction for a period of 10–100 s. The CO₂ concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition constants. For each inhibitor, at least six traces of the initial 5–10% of the reaction were used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitor (1 mM) were prepared in distilled-deionized water with 10–20% (v/v) DMSO (which is not inhibitory at these concentrations), and dilutions up to 0.01 nM were done thereafter with distilled-deionized water. Inhibitor and enzyme solutions were preincubated together for 15 min at room temperature prior to the assay, to allow for the formation of the E-I complex. The inhibition constants were obtained by non-linear least-squares methods using PRISM 3 from Lineweaver-Burk plots, as reported earlier, and represent the mean from at least three different determinations.

Real-time PCR Experiments—Real-time PCR was performed on the following genes of *S. pistillata*: STPCA (cloned in the present study, GenBank™ accession no. EU159467), STP36B4 (Ref. 23, GenBank™ accession no. EU069460), and β -actin (Ref. 33, accession no. AY360081). Specific primers of STPCA: forward (5'-GGACATACCGTTCAGGCGACTTTT-3') and reverse (5'-TTTCTTCCACCGACCTGATGCTCT-3'); STP36B4: forward (5'-ATGCAGCAAATCCGTCAGTCTCT-3') and reverse (5'-TGGCCTTTCTTATCATGGTGTCT-3'); and β -actin: forward (5'-GACTTGGCCGGCAGGGACTTA-3') and reverse (5'-GCTCGGCGGTTGTGGTGAAA-3') were designed using the software Primer express (PerkinElmer/Applied Biosystems Divisions). PCR efficiency was calculated according to the equation $E = 10^{-1/\text{slope}} - 1$ where maximal efficiency, $E = 1$ (100%), means that every single template is replicated in each cycle.

Real-time PCR was performed using the Prism 7300 sequence detector and 2 \times SYBR® Green Master Mix (Applied Biosystems Divisions) in triplicate in a 96-well optical plate (PerkinElmer/Applied Biosystems Divisions) in 20:5 μl of cDNA (diluted 1:10), 0.2 μl each of primers (10 μM), 10 μl of Mix SYBRgreen, and 4.6 μl of RNase-free water. PCR was performed with the following PCR parameters: 50 °C for 2 min, 95 °C for 10 min, then 95 °C for 15 s, 60 °C for 1 min for 40 cycles plus a dissociation step (60–95 °C). RNA transcription levels were determined by the method of direct comparison of C_T values between target (STPCA) and reference (STP36B4 and β -actin) genes (23) and relative quantities calculated by the $\Delta\Delta C_T$ equation with the light value as calibrator. Finally, data

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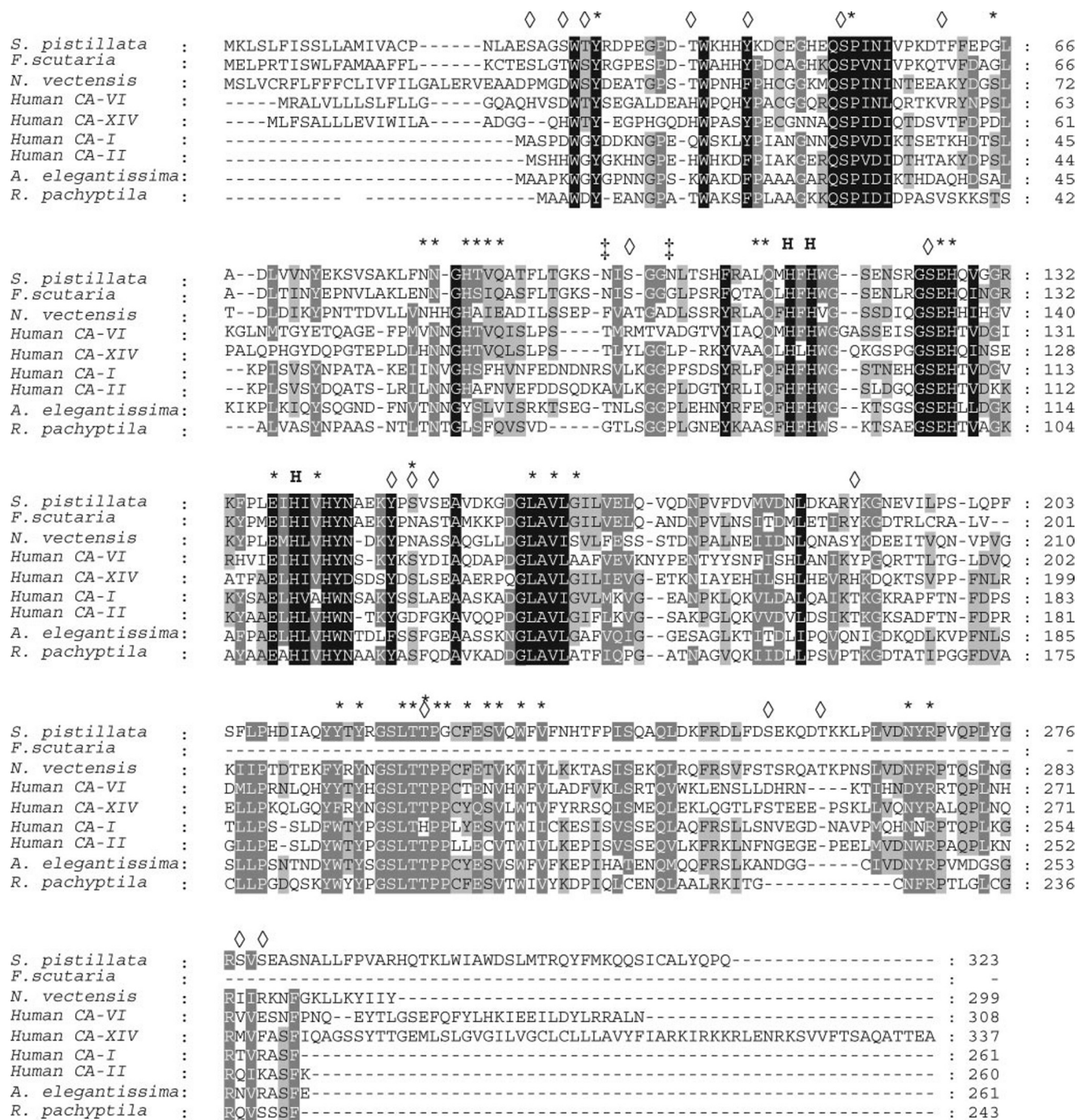


FIGURE 1. Alignment of α -CA sequences from *S. pistillata* (STPCA), *F. scutaria* (FCAa), *N. vectensis*, *Homo sapiens* CAVI, XIV, I, II, *A. elegantissima*, and *R. pachyptila*. Conserved amino acids (AA) are in black, AA identical (80%) are in dark gray, AA identical (60%) are in light gray. H represents histidine residues essential for zinc cofactor binding, and asterisks mark the 33 important AA involved in the hydrogen-bond network. The 23 first AA of the STPCA correspond to the signal peptide. The putative N-glycosylation sites are shown as ‡, and putative phosphorylation sites as ◇.

were transformed into linear form by $2^{-\Delta\Delta CT}$ (34). Data representative of four independent experiments are presented. Statistical analysis was performed using a Student's *t* test (Software Jump 5.1, Cary, NC). Results are considered statistically significant when $p < 0.05$.

RESULTS

Cloning of a Carbonic Anhydrase from the Coral S. pistillata—PCR using degenerate primers provided a *S. pistillata* partial

sequence. A GenBankTM comparison using BLASTX confirmed that the partial sequence derived from *S. pistillata* corresponds to a carbonic anhydrase. RACE experiments were used to obtain full-length cDNA. The complete sequence consists of 1542 bp long with a 972-nucleotide open reading frame beginning 97 nucleotides downstream from the 5'-end. This sequence codes for a protein of 324 amino acids and a calculated molecular mass of 36.6 kDa. The nucleotide sequence of the STPCA is available on the GenBankTM data base with accession number EU159467.

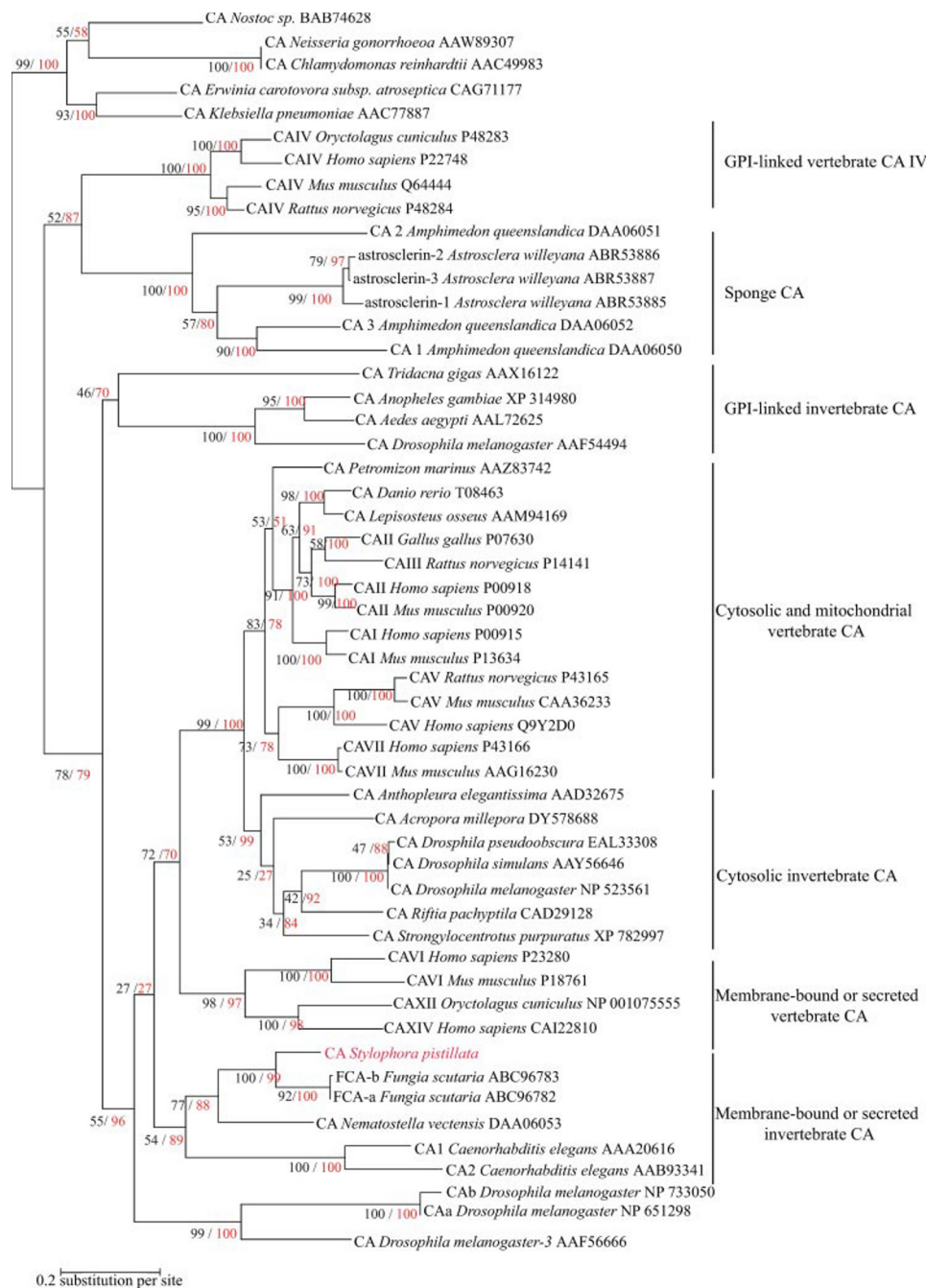


FIGURE 2. **Phylogenetic relationships of vertebrates and invertebrates α -CA sequences inferred from ML and Bayesian analyses.** Bootstrap network of 53 α -CA gene sequences based on ML distances are estimated with a WAG +G+F model ($\alpha = 0.193$) using PHYML. Bootstrap values were computed from 100 replications starting from both a BIONJ tree (dark) and the tree inferred by MrBAYES (red).

Sequence Analysis—BLAST searches in GenBankTM reveal numerous significant alignments with vertebrate and invertebrate α -CA sequences. A conserved domain search on NCBI confirmed the presence of the carbonic anhydrase catalytic domain containing the three histidine residues essential for the zinc cofactor binding noted by an *H* in Fig. 1, and the thirty-three important amino acids involved in the hydrogen bond network noted by an *asterisk* in Fig. 1.

A signal peptide is present in the STPCA sequence, with predicted cleavage between positions 23 and 24, indicating that the

sequence is probably secreted or membrane-bound. Because no predicted GPI anchor and no transmembrane domain such as a hydrophobic α -helix or β -sheet could be detected, we suggest that the protein STPCA is a secreted form of CA. Other particular motifs are also present in the STPCA sequence: two *N*-glycosylation sites in positions 98 and 103, and seventeen predicted phosphorylation sites (10 serine, 5 threonine, and 3 tyrosine residues).

Phylogenetic Tree of α -CA Sequences—ML and Bayesian method trees produced similar topologies. Bootstrap values for ML and MR Bayes are shown near the recurrent nodes in Fig. 2. Fifty-three sequences are included in this tree, rooting with the α -CA from the cyanobacterium *Nostoc sp.* (GenBankTM accession number BAB74628). The STPCA sequence groups with the sequences of two cnidarians: *Nematostella vectensis* and aposymbiotic larvae of *Fungia scutaria*.

Immunolocalization—To localize the STPCA enzyme in coral, tissue sections were immunolabeled with the anti-STPCA antibody (Fig. 3). *S. pistillata* is a colonial coral characterized by the presence of numerous polyps, linked together by a common tissue usually referred to as the coenosarc. The tissue in contact with seawater is the oral whereas the tissue adjacent to the skeleton is the aboral tissue. The tissues consist of two epithelial layers named ectoderm and endoderm. The aboral ectoderm is a specialized tissue that is in contact with the skeleton. This calcium carbonate-producing cell layer is often referred to as the skeletogenic tissue, the calicoblastic ectoderm (for histology details see Refs. 31, 35). Fig. 3A shows a section of *S. pistillata* tissue in the coenosarc region with the oral tissue facing the seawater and the aboral tissue facing the skeleton. Fig. 3B is a magnification of the aboral tissue and shows the labeling of the calicoblastic ectoderm. No staining was observed in controls using preimmune serum (Fig. 3C).

Biochemical Characterization of the Carbonic Anhydrase—A recombinant form of the carbonic anhydrase was produced in HEK 293 cells. The chimeric form with a polyhistidine tag at the

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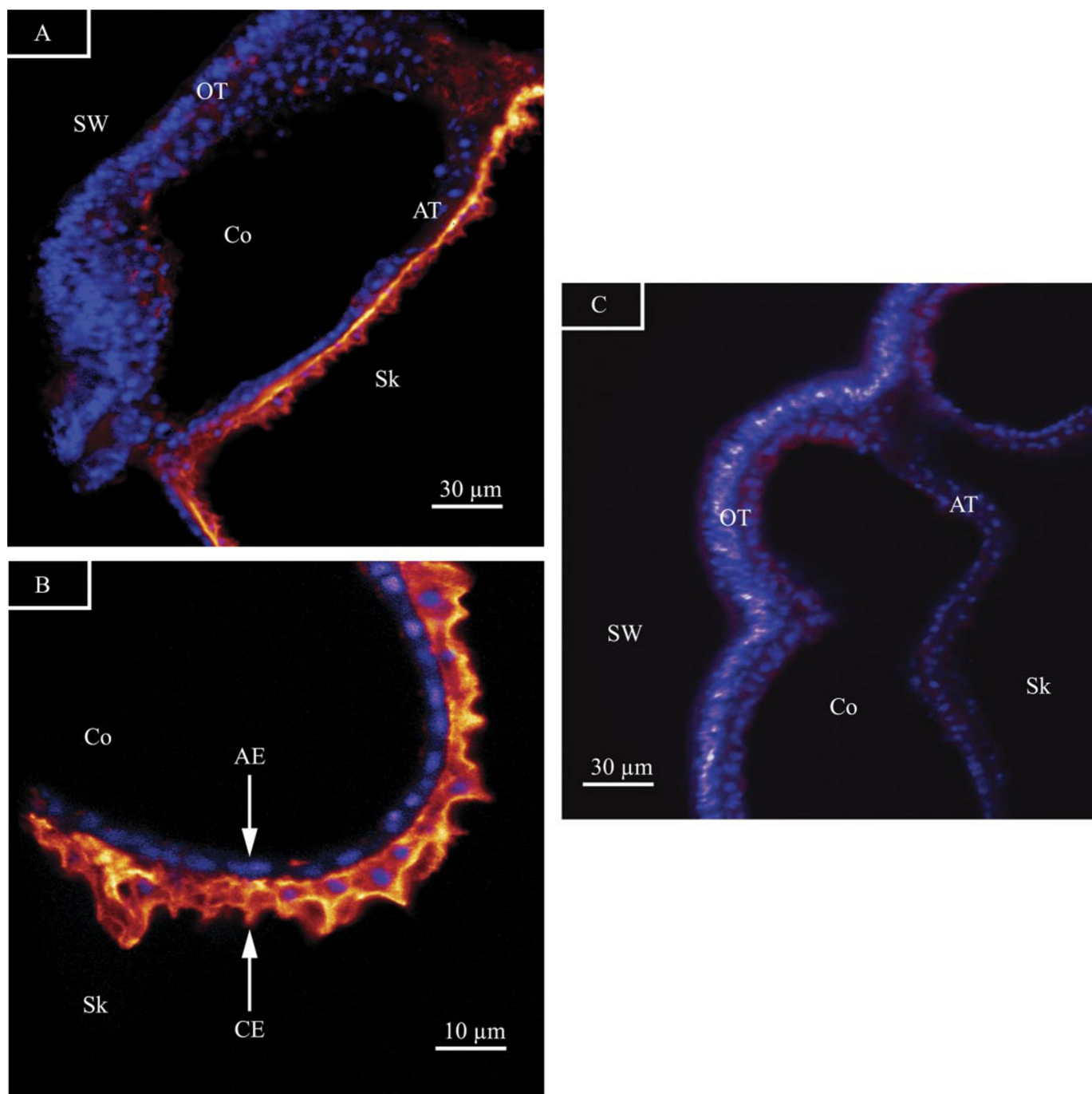


FIGURE 3. Immunolocalization of α -STPCA on the tissue slide of *S. pistillata* with carbonic anhydrase-specific antibody (anti-STPCA) or preimmune serum. Anti-STPCA coupled to Alexafluor 568 is shown in orange, merged with DAPI staining showing the nuclei in blue. A, large view of oral tissue (OT) and aboral tissue (AT) of coral. B, magnification of the aboral tissue showing the aboral endoderm (AE), and the calcicoblastic ectoderm (CE). C, control with preimmune serum. Other abbreviations: Co, coelenteron; Sk, skeleton; SW, seawater.

C terminus was harvested in the culture medium and purified on NTA beads. Then STPCA was submitted to *N*-glycanase (*N*-PGNase) and migrated on SDS-PAGE. A Western blot (Fig. 4A) with anti-STPCA or antihistidine tag was performed. The glycosylated protein possesses an apparent molecular mass of about 44 kDa, whereas the *N*-PGNase-treated protein is 36.6 kDa as predicted by the primary sequence. In the same manner, the recombinant STPCA was submitted to a Western blot with anti-phosphoserine, anti-phosphothreonine, or anti-phospho-

tyrosine. Results show that only serines are phosphorylated on the molecule (Fig. 4B).

CA activity and inhibition assays were performed on the recombinant STPCA. The kinetic parameters for the CO_2 hydration reaction by the STPCA, as well as for the human isoforms are shown in Table 1. The results show that the STPCA has a lower catalytic activity than hCAII, but of the same order of magnitude as that of the cytosolic isoform hCAI. In fact, this activity is very similar to the hCAVI, which is also a

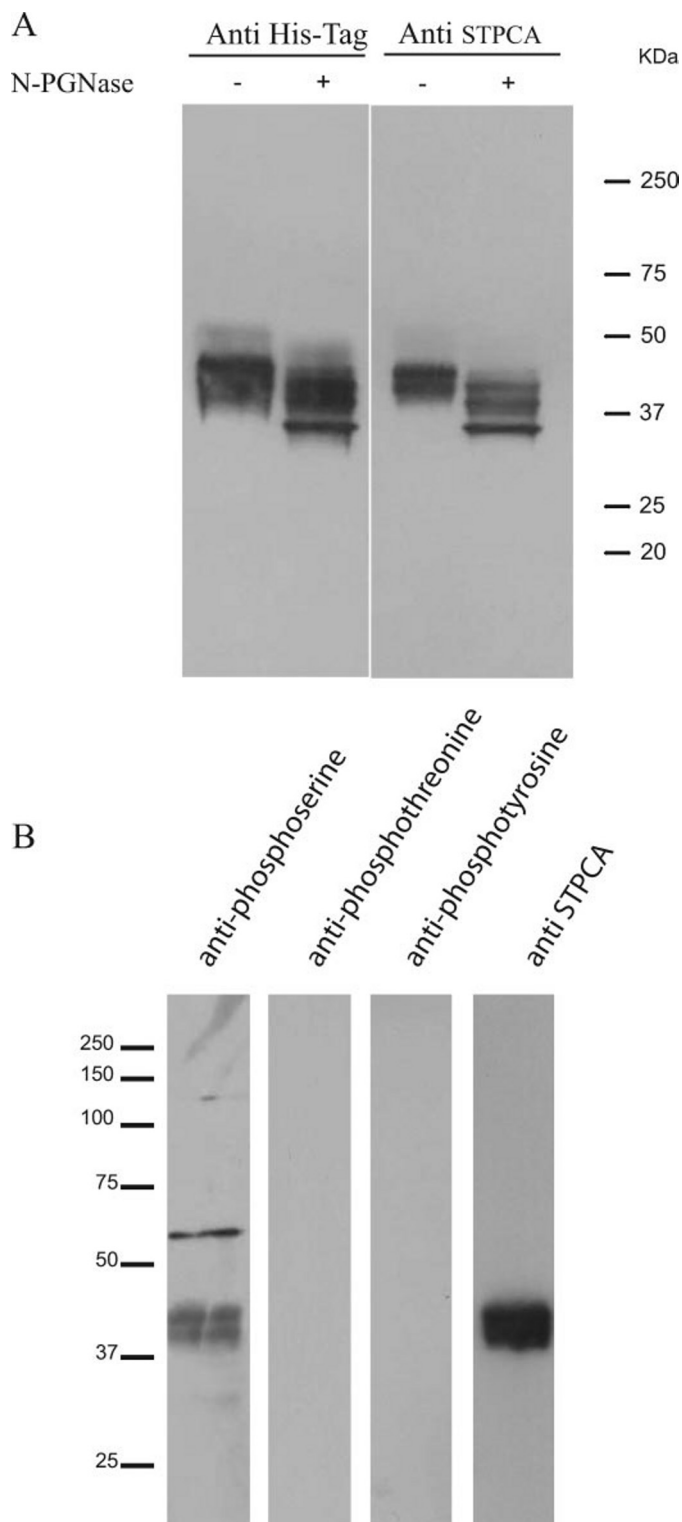


FIGURE 4. Western blot showing that A/STPCA protein is glycosylated. After *N*-glycosidase F (*N*-PGNase) treatment, recombinant STPCA protein was subjected to SDS-PAGE, electroblotted, and incubated with anti-histidine tag or a specific anti-STPCA; B/STPCA is phosphorylated on serine: recombinant STPCA was subjected to SDS-PAGE, electroblotted, and incubated with anti-phosphoserine, anti-phosphothreonine, anti-phosphotyrosine, or anti-STPCA as control.

secreted isoform. As shown in Table 1, hCAI, hCAVI, and STPCA have a very similar k_{cat} and a near-similar k_{cat}/K_m , close to $5.0 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$. It may also be observed from the data of

TABLE 1
Kinetic parameters for the CO_2 hydration reaction

Shown are kinetic parameters for the CO_2 hydration reaction (32) catalyzed by the human cytosolic α -hCA isozymes I-III, the mitochondrial isozymes hCA VA and VB, the secreted isoform hCA VI (full-length), and the transmembrane isozymes hCA IX (catalytic domain), hCA XII (catalytic domain), and the STPCA at 20 °C and pH 7.5 in 10 mM HEPES buffer, and their inhibition data with acetazolamide (5-acetamido-1,3,4-thiadiazole-2-sulfonamide) (53), a clinically used drug.

| Isozyme | Activity level | k_{cat} | k_{cat}/K_m | K_I (acetazolamide) |
|---------|----------------|------------------|-------------------------------------|-----------------------|
| | | s^{-1} | $\text{M}^{-1} \cdot \text{s}^{-1}$ | mM |
| hCA I | Moderate | $2.0 \cdot 10^5$ | $5.0 \cdot 10^7$ | 250 |
| hCA II | Very high | $1.4 \cdot 10^6$ | $1.5 \cdot 10^8$ | 12 |
| hCA III | Very low | $1.0 \cdot 10^4$ | $3.0 \cdot 10^5$ | 300,000 |
| hCA VA | Low | $2.9 \cdot 10^5$ | $2.9 \cdot 10^7$ | 63 |
| hCA VB | High | $9.5 \cdot 10^5$ | $9.8 \cdot 10^7$ | 54 |
| hCA VI | Moderate | $3.4 \cdot 10^5$ | $4.9 \cdot 10^7$ | 11 |
| hCA IX | High | $3.8 \cdot 10^5$ | $5.5 \cdot 10^7$ | 25 |
| hCA XII | Moderate | $4.2 \cdot 10^5$ | $3.5 \cdot 10^7$ | 5.7 |
| STPCA | Moderate | $3.1 \cdot 10^5$ | $4.6 \cdot 10^7$ | 16 |

Table 1 that STPCA is highly inhibited by acetazolamide, similar to hCA II and hCAVI.

Real-time PCR Experiments—To determine the role of STPCA in the “light-enhanced calcification” phenomenon, expression levels of the STPCA gene were measured by real-time PCR under light and dark conditions. Results are reported as relative expression with the genes STP36B4 and β -actin as references (23), and the value obtained in the light condition as a calibrator. PCR efficiency, calculated according to the equation $E = 10^{-1/\text{slope}} - 1$, reveals 100% (slope: -3.32), 97.2% (slope: -3.39), and 93.1% (slope: -3.50) for the amplification of STPCA, STP36B4, and β -actin genes, respectively. The absence of nonspecific products was confirmed by dissociation curve analysis. Results show that in four independent experiments, the expression level of STPCA is higher during the dark (1.97 ± 0.11) than during the light period (1.00 ± 0.00) (Student's *t* test, $p < 0.05$).

DISCUSSION

A Carbonic Anhydrase from the Coral *S. pistillata*—Using degenerate primers designed on the α -CA sequences, we cloned and sequenced a carbonic anhydrase in the scleractinian coral *S. pistillata*. This α -CA, named STPCA, shows a sequence of 324 amino acids (Fig. 1). The STPCA contains a complete CA catalytic site composed of three histidine residues that bind the zinc cofactor, and 33 amino acids involved in the hydrogen-bond network, as predicted for functional CAs (1). To date, only two entire coral CA sequences have been cloned, both in the coral *F. scutaria* (36). These two sequences, named FCA *a* and *b*, contain 201 and 225 amino acids, respectively (36). Both sequences are shorter than the STPCA sequence. The FCA *a* and *b* sequences revealed a truncated catalytic site containing the three histidine residues but lacking, respectively, 14 and 6 amino acids from the C terminus (36).

Similar to FCA *a* and *b*, the presence of a signal peptide indicates that the STPCA protein is not cytosolic but secreted or membrane-bound. The sequence STPCA does not contain a predicted GPI anchor site or transmembrane domain, suggesting that this protein is secreted rather than membrane-bound.

Phylogenetic Positioning of the STPCA Sequence—The BLAST search in GenBank™ indicated that the STPCA

Carbonic Anhydrase in the Coral *S. pistillata*

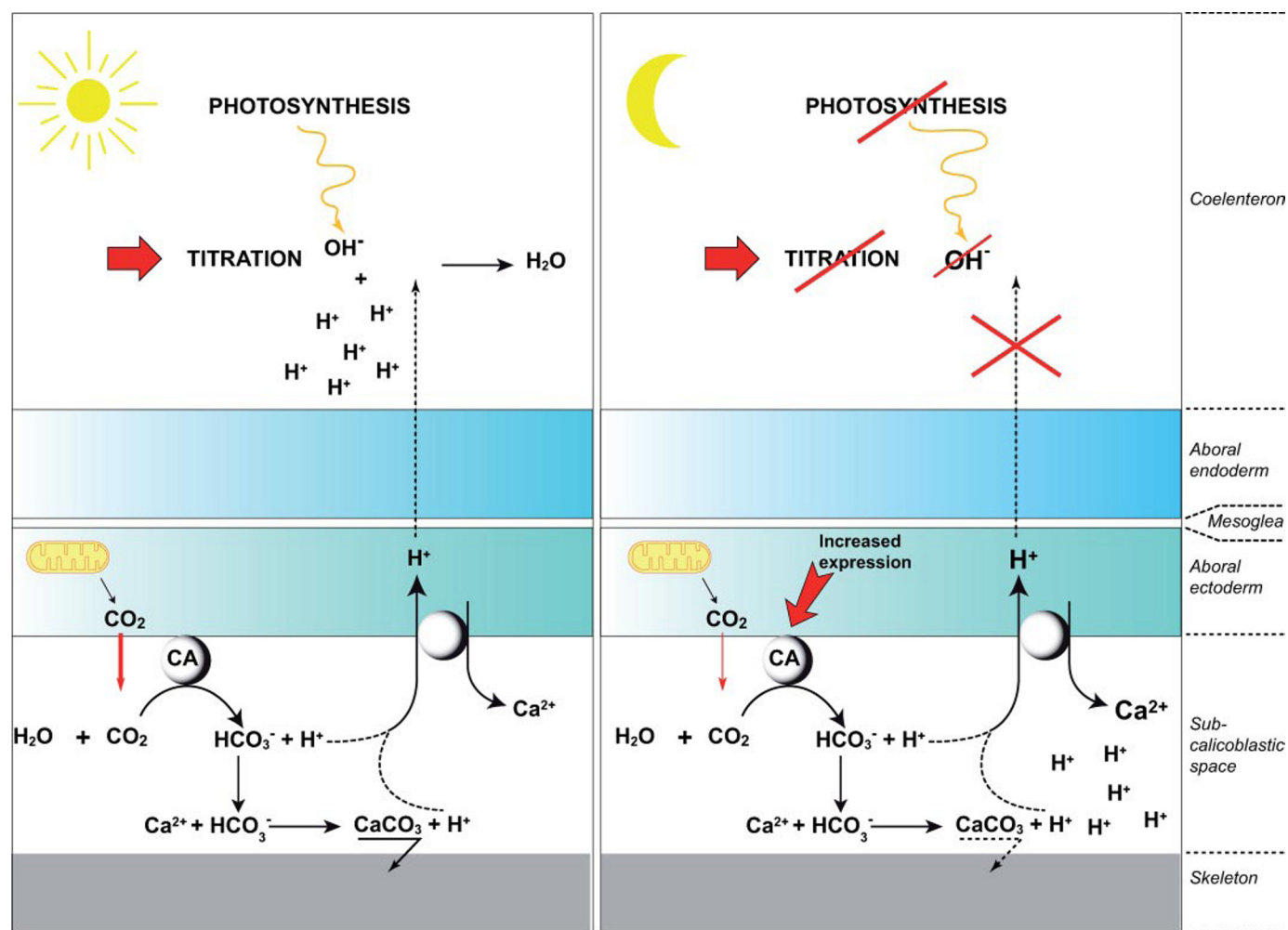


FIGURE 5. **Schematic model proposed for the function of CA in the calcification process.** During the light, protons produced by calcification are titrated by the alkaline environment because of the photosynthetic process, which facilitates CO_2 diffusion and thus calcium carbonate precipitation. During the dark, protons are not titrated and tend to be accumulated in the subcalicoblastic space and the surrounding tissues, which decrease CO_2 diffusion from calcicoblastic cells and thus calcification. We suggest that an up-regulation of STPCA expression during the dark allows coping with night acidosis.

sequence belongs to the class α of carbonic anhydrases. Thus, we constructed a phylogenetic tree with 53 α -CA sequences (Fig. 2). This phylogenetic reconstruction indicates that the STPCA sequence groups with those of the cnidarians *F. scutaria* and *N. vectensis*. Sequences of *F. scutaria* have been isolated from aposymbiotic larvae, and *N. vectensis* does not establish symbiosis with dinoflagellates, indicating that these sequences belong specifically to cnidarians. We can thus confirm that the STPCA belongs to the host cnidaria and not to the symbiotic dinoflagellates of *S. pistillata*.

The phylogenetic tree is in accordance with the results of Jackson *et al.* (37). By phylogenetic reconstruction, these authors have shown that the α -CA family expanded from a single ancestral gene through several independent gene duplication events in sponges and eumetazoans (37). In contrast to Jackson *et al.*, we found that vertebrate CAsIV forms a sister group with the ancestral sponge CAs. Even if currently we do not have any explanation, this result indicates that CAsIV diverged earlier than the other eumetazoan sequences. One specific feature of CAsIV is that they are characteristically attached to membranes via a GPI tail.

The *S. pistillata* sequence belongs to a group containing invertebrate membrane-bound or secreted sequences (*N. vectensis*, *F. scutaria* larvae), confirming that STPCA is not a cytosolic form of CA. Sequences of *N. vectensis* and *F. scutaria* larvae do not contain a predicted GPI anchor, similar to STPCA, suggesting that this group has lost a potential GPI anchor that was present in former sequences.

Fig. 2 shows that membrane-bound/secreted invertebrates CAs diverged before the appearance of cytosolic (vertebrates and invertebrates) and membrane-bound/secreted vertebrates CAs. Finally, there is a split between invertebrate and vertebrate cytosolic sequences. Indeed, the sequences from the invertebrates *Anthopleura elegantissima*, *Strongylocentrotus purpuratus*, and *Riftia pachyptila*, are all described in the literature as cytosolic forms of CA (38–40). This group also contains a coral CA that was recovered bioinformatically from the EST data base of *Acropora millepora* (41).

As shown in the phylogenetic reconstruction of Jackson *et al.* (37), we confirmed that the ancestor of carbonic anhydrases is a membrane-bound or secreted form, which then diverged progressively into cytosolic forms. Hewett-Emmett and Tashian

(1) already suggested that transmembrane, membrane-associated, and extracellular CAs form a clade that branch earlier than the cytosolic CA form.

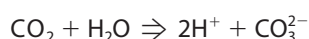
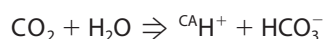
The membership of STPCA to the secreted form of CA is confirmed by CA activity and inhibition assay experiments. Indeed, the STPCA has an activity similar to that of secreted hCA VI (Fig. 4A), *i.e.* STPCA is moderately active as a CO₂ hydration catalyst but is highly inhibited by acetazolamide, similar to hCA VI (Fig. 4B).

Role of CA in the Calcification Process—In symbiotic Cnidarians, such as *S. pistillata*, CA has been shown to play a role in both CO₂ supply to symbiont photosynthesis (42–44) and calcification (7, 14, 15–17). CA cloned in the present study appears to be linked only to the second process because: 1) it is only located within the calcicoblastic epithelium, away from zooxanthellae (Fig. 3), and 2) similar sequences were observed in symbiotic and non-symbiotic Cnidarians.

The first evidence of the role of CA in coral calcification dates from the study of Goreau (7). As the CA inhibitor Diamox reduced calcification rates, this author suggested that the enzyme helps in removing carbonic acid produced by calcification from the skeletogenic site. Since then, numerous authors have also observed that CA inhibitors decrease calcification rates and have suggested that a CA is involved in the inorganic carbon supply for calcification and/or the regulation of pH at the calcification site (14, 15–17).

A direct role for STPCA in calcification is confirmed by the specific localization of this enzyme in the calcicoblastic ectoderm, which is the epithelium facing the skeleton. Indeed, it is well accepted that this epithelium plays a key role in calcification (31, 35, 45). Its localization is in agreement with the results of Isa and Yamazato (12) who localized histochemically CA activity in the calcicoblastic ectoderm of the coral *Acropora hebes*. Similarly, in the gorgonian *Leptogorgia virgulata*, the CA activity was preferentially localized in the scleroblasts and axial epithelium, precisely on the membrane of the spicule-forming vacuole (13), again suggesting an important role of CA in the calcification process. This does not preclude, however, the presence of at least another CA isoform for CO₂ supply to symbiont as previously suggested (42, 44, 46).

The localization of STPCA together with the sequence analysis and phylogenetic position of the STPCA gene suggest that the enzyme is secreted by the calcicoblastic cells. It has been shown by Erez (47) and Furla *et al.* (15) that metabolic CO₂ is the major source of carbon for calcification. In this case, the secreted CA may help in converting this CO₂ (which diffuses from the tissue to the skeletogenic fluid because of the high pH of this calcifying region (48)) into HCO₃⁻, following Reaction Scheme 1.



REACTION SCHEME 1

The two H⁺ produced by this set of reactions may then be removed from the site of calcification by the Ca²⁺-ATPase

present within the calcicoblastic epithelium (33), which catalyzes the exchange 2H⁺/Ca²⁺.

Light-enhanced Calcification—Since 1948, it is well known that light influences calcification in corals (49). Under our culture conditions, we have shown that the day calcification rate is 2.6 times higher than the dark calcification rate in *S. pistillata* (21). This enhancement of calcification by light is due to the symbiosis between corals and their photosynthetic dinoflagellates symbionts, but its mechanism remains largely unknown (see review in Refs. 19, 20).

In the present study, we investigated the differential expression of the STPCA gene during light and dark conditions. Our results show that the STPCA gene is up-regulated during the dark ~2-fold compared with light (Student's *t* test < 0.05), suggesting a correlative increase in protein concentration and thus in CA activity. It is important to notice that it is the first time that such a daily pattern is observed in corals for a CA gene as well as for any other genes. A similar light/dark regulation was previously observed in fish otoliths in which CA shows a marked diurnal variation of gene expression with ~4-fold increase of expression at night compared with morning expression (11). Otolith growth follows a daily rhythm with alternative organic-rich layers and mineral-rich layers, respectively, during the night and day, but parameters controlling this rhythm are not well understood (see review in Refs. 50, 51). To explain the differential expression of CA between light and dark, Tohse *et al.* (11) suggested that CA might contribute to the mineralization process of otoliths by controlling the inorganic environment and the diurnal variations of pH in the endolymph.

In corals, previous measurements of pH at the subcalicoblastic and coelenteron levels showed an acidification during dark compared with light conditions (48, 52). Indeed, during the dark, protons produced by calcification cannot be titrated within the coelenteron by the alkaline environment since there is no photosynthesis (15). In the coral *S. pistillata*, we suggest (Fig. 5) that the up-regulation of STPCA expression during the dark facilitates the continuous production of bicarbonate from metabolic CO₂ despite an increase in H⁺ concentration into the subcalicoblastic environment (from 9.3 in the light to 8.2 in the dark (16)). Such a result is in agreement with those of Goreau (7) and Furla *et al.* (15) who showed that the effect of carbonic anhydrase inhibitors on calcification is higher in the dark than in the light, suggesting an increase of carbonic anhydrase activity in the dark. This result also shows that despite an enhancement of CA expression during the dark, a change in CA expression is likely not to be involved in light-enhanced calcification, which seems to be based at least in part on a more favorable pH environment in the light (15).

In summary, the STPCA cloned in this study is a secreted enzyme whose synthesis by the calcicoblastic ectoderm suggests a role in calcification. We propose that this CA increases the hydration rate of metabolic CO₂ into HCO₃⁻. This is the first report to show an up-regulation of a coral gene during a diurnal period. This up-regulation of STPCA may then help to sustain the CO₂ hydration rate during dark acidic conditions.

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