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CHARACTERIZATION OF A FUNCTIONAL VANADIUM-DEPENDENT BROMOPEROXIDASE IN THE MARINE CYANOBACTERIUM *SYNECHOCOCCUS* SP. CC9311[1]

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Vanadium-dependent bromoperoxidases (VBPOs) are characterized by the ability to oxidize halides using hydrogen peroxide. These enzymes are well-studied in eukaryotic macroalgae and are known to produce a variety of brominated secondary metabolites. Though genes have been annotated as VBPO in multiple prokaryotic genomes, they remain uncharacterized. The genome of the coastal marine cyanobacterium *Synechococcus* sp. CC9311 encodes a predicted VBPO (YP_731869.1, sync_2681), and in this study, we show that protein extracts from axenic cultures of *Synechococcus* possess bromoperoxidase activity, oxidizing bromide and iodide, but not chloride. In-gel activity assays of *Synechococcus* proteins separated using PAGE reveal a single band having VBPO activity. When sequenced via liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS), peptides from the band aligned to the VBPO sequence predicted by the open reading frame (ORF) sync_2681. We show that a VBPO gene is present in a closely related strain, *Synechococcus* sp. WH8020, but not other clade I *Synechococcus* strains, consistent with recent horizontal transfer of the gene into *Synechococcus*. Diverse cyanobacterial-like VBPO genes were detected in a pelagic environment off the California coast using PCR. Investigation of functional VBPOs in unicellular cyanobacteria may lead to discovery of novel halogenated molecules and a better understanding of these organisms' chemical ecology and physiology.

Key index words: **cyanobacteria; horizontal gene transfer; specific activity; *Synechococcus*; vanadium-dependent bromoperoxidase**

Abbreviations: **GOS database, Global Ocean Sampling database; HGT, horizontal gene transfer; MCD, monochlorodimedone; PhR, phenol red; ThB, thymol blue; VBPO, vanadium-dependent bromoperoxidase**

Vanadium-dependent haloperoxidases are found in a wide range of organisms and are well-studied for their ability to oxidize halides (Winter and Moore 2009). A subset of these halogenating enzymes, VBPOs, has been predominantly identified and characterized in eukaryotic macroalgae (Ohsawa et al. 2001, Carter et al. 2002, Kamenarska et al. 2007), though they have been suggested to be present in prokaryotes. The reaction mechanism of VBPO from the rhodophyte *Corallina officinalis* has been described by Carter et al. (2002). The enzyme first binds to a molecule of hydrogen peroxide. The peroxo-bound form of the enzyme carries out the two-electron oxidation of bromide, forming hypobromous acid. This reactive intermediate attaches bromine to organic molecules through an electrophilic reaction. The hypobromous acid intermediate is generally thought to be freely dispersing. However, strong evidence supports specificity toward a final organic substrate (Butler and Carter-Franklin 2004, Kamenarska et al. 2007, Hartung et al. 2009).

VBPOs have two proposed functions in vivo. Primarily, the enzyme is associated with the ability to synthesize a variety of brominated organic compounds, thought to serve as an allelopathic defense. In eukaryotic macroalgae, bromoform production prevents growth of epiphytic diatoms (Ohsawa et al. 2001), whereas brominated compounds in rhodophytes have antimicrobial and other bioactive properties (Butler and Carter-Franklin 2004). Though an active bromperoxidase has not been characterized in a cyanobacterium, brominated compounds are produced by some groups. The first demonstration of halogenated molecules in cyanobacteria was the finding of brominated phenols in *Calothrix brevissima* (Pedersén and DaSilva 1973). Other studies have also shown that polybrominated diphenyl ethers are produced by *Oscillatoria*, a sponge endosymbiont (Unson et al. 1994, Agrawal and Bowden 2005). Monohalomethane and polyhalomethane production has been associated with the cyanobacterial fraction of a bloom in the Baltic Sea, in which *Pseudoanabaena* sp. was the dominant species along with other filamentous cyanobacteria (Karlsson et al. 2008). Halogenated compounds could serve as an allelopathic defense for marine *Synechococcus,* which is subject to high grazing pressure from eukaryotes and competition from heterotrophic bacteria (Caron et al. 1991, Strom 2002).

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VBPO also has a possible physiological role as an antioxidant. Hydrogen peroxide, a metabolic by-product of photosynthesis, is a damaging reactive oxygen species that is present in seawater up to nanomolar concentrations (Drabkova et al. 2007). VBPO reduces hydrogen peroxide to water with the oxidation of bromide. Manley and Barbero (2001) explored the role of VBPO in the remediation of photosynthetically produced H_2O_2, finding a correlation between bromoperoxidase activity in the chlorophyte *Ulva lacta* and light and dark cycles. Another study found that vanadium chloroperoxidase was down-regulated under light deprivation in the transcriptome of *Gracilaria algae* (Ho et al. 2009). VBPO could act as an antioxidant system for marine cyanobacteria, decreasing internally produced H_2O_2; however, VBPOs have been primarily found and studied in eukaryotic macroalgae.

Synechococcus is a genus of unicellular chroococcoid cyanobacteria that contributes up to 20%–30% of photosynthetically fixed marine primary production (Li 1994, Jardillier et al. 2010). Due to the abundance and global distribution of *Synechococcus*, it is important to understand the ecology and physiology of these photoautotrophs. As a primary producer, *Synechococcus* is subject to high predation rates by heterotrophic plankton (Christaki et al. 2002, Chan et al. 2009). An increasing number of isolated *Synechococcus* strains from different clades (species) with available whole genome sequences provide an opportunity to investigate physiological differences at the clade and strain levels, as well as address questions such as the role of horizontally transferred genes. At least 10 distinct clades of *Synechococcus* have been observed (Dufresne et al. 2008), based on both internal transcribed spacer (ITS) and 16S rRNA sequences, demonstrative of the fine-scale diversity within this genus.

To date, the presence of VBPO in prokaryotic photoautotrophs has only been inferred from genome sequencing. A VBPO was recently annotated in the genome of *Synechococcus* sp. CC9311 (Palenik et al. 2006) and could have been acquired by horizontal gene transfer (HGT), supported by the fact that it is found in an island of genes unique to *Synechococcus* sp. CC9311 among sequenced *Synechococcus* genomes (Dufresne et al. 2008), with many of the island's genes showing atypical trinucleotide composition (Palenik et al. 2006). Putative bromoperoxidases are present in several other sequenced cyanobacterial genomes (Swingley et al. 2008; *Crocosphaera watsonii* WH8501, GenBank AADV00000000.2, *Synechococcus* sp. PCC 7335, GenBank ABRV00000000). However, the distribution, activity, and function of this enzyme among cyanobacteria have remained undefined.

We report here on the first characterization of a functional bromoperoxidase in a cyanobacterium and investigate the distribution of bromoperoxidase genes in strains of marine *Synechococcus* and in the pelagic marine environment.

MATERIALS AND METHODS

Strains and culture conditions. *Synechococcus* sp. used in this study include strains CC9311 (Palenik et al. 2006, CCMP2515), WH8102 (Waterbury et al. 1986, CCMP2370), WH8020 (Waterbury et al. 1986), CC9617 (Toledo et al. 1999), and WH8016 (Waterbury et al. 1986). All stock cultures were maintained in SN medium (Waterbury and Willey 1988) prepared using seawater from the Scripps Pier (La Jolla, CA; 32°52′ N, 117°15.4′ W). For assays, cultures were grown at constant light ($\sim$20 $\mu E \cdot m^{-2} \cdot s^{-1}$) at 18°C with stirring. Cultures of *Synechococcus* sp. CC9311 and WH8102 were axenic, whereas *Synechococcus* sp. WH8020, CC9617, and WH8016 were not axenic.

Protein extractions. Proteins were collected from *Synechococcus* cultures in late exponential growth. Cell pellets from 1 L cultures were suspended in 3 mL 100 mM 2(N-morpholino) ethanesulfonic acid (MES) buffer, pH 7.0, and broken using a French pressure cell with three passes at 20,000 psi. Unbroken cells were removed by centrifugation (Sorvall RC28S, Thermo Fisher Scientific, Waltham, MA, USA) at 6,278*g* for 10 min at room temperature. The supernatant, consisting of soluble and membrane-bound fractions, was taken as crude protein extract. Subsequent centrifugation at 100,446*g* for 90 min (4°C) was performed to separate membrane from soluble proteins (Brahamsha 1996). The membrane fraction pellet was washed with 3 mL of 100 mM MES buffer, pH 7.0, and collected by centrifugation for 90 min at 100,446*g*. All proteins were handled on ice and stored at −80°C until use. Protein concentrations were determined using the Pierce BCA Protein Assay (Thermo Fisher Scientific, Rockford, IL, USA) with BSA standards.

Enzyme activity. Protein extracts from *Synechococcus* cultures were tested for bromoperoxidase activity using monochlorodimedone (MCD; Sigma Inc., St. Louis, MO, USA) and thymol blue (ThB; ACROS, Geel, Belgium), as described in Carter et al. (2002), Kamenarska et al. (2007), and Verhaeghe et al. (2008) with the following modifications. Crude protein extracts were incubated with sodium orthovanadate at a final concentration of 1.98 mM at room temperature for 20 min prior to the assay. For the MCD assay, crude, soluble, and membrane-bound protein extracts (0.05–0.1 $mg \cdot mL^{-1}$ final conc.) were added to 100 mM KBr in 100 mM MES buffer (pH 7.0) and 60 μM MCD. The reaction was started with the addition of hydrogen peroxide to a final concentration of 2 mM and monitored spectrophotometrically at 290 nm (MCD, $\varepsilon = 19.9\ mM^{-1} \cdot cm^{-1}$). In the second bromoperoxidase assay, 10 μM ThB (final concentration) replaced MCD. The absorbance of the dihalogenated product was monitored at 620 nm ($ThBI_2$, $\varepsilon = 40.3\ mM^{-1} \cdot cm^{-1}$; $ThBBr_2$, $\varepsilon = 37.2\ mM^{-1} \cdot cm^{-1}$). Specific activities are reported as μmol substrate brominated $\cdot$ $min^{-1} \cdot mg\ protein^{-1}$.

Halide specificity, kinetics. We tested halide specificity by substituting potassium bromide (1–100 mM) with either potassium iodide (0.5–50 mM) or potassium chloride (100 mM) in the reaction. We used MCD assays to compare bromide oxidation with chloride oxidation in crude extracts. We used ThB, however, to compare VBPO oxidation of bromide and iodide. We also measured the ability of VBPO in crude extracts to oxidize iodide by measuring the accumulation of triiodide at 350 nm in the presence of hydrogen peroxide and iodide as described by Kamenarska et al. (2007).

The reaction velocities for the enzyme were determined over the first minute at each concentration, then fitted to a Michaelis–Menten, nonlinear regression using GraphPad Prism software version 5.0b (GraphPad Software, San Diego, CA, USA).

SDS-PAGE. To identify the proteins responsible for the observed activity, polyacrylamide gels were run under native and denaturing conditions, and then stained for bromoperoxidase activity. For native conditions, *Synechococcus* protein extracts were loaded on Novex Nupage 7% Tris-Acetate gels (Invitrogen, Carlsbad, CA, USA) and run with Tris-Glycine Native Running buffer consisting of 25 mM Tris-base and 192 mM glycine, pH 8.3 (Invitrogen) at 150 V for 2 h. For denaturing conditions, protein extracts were incubated at 70°C for 10 min in the presence of Novex Reducing Agent (Invitrogen) following the manufacturer's recommendations and run with Tris-Acetate SDS Running Buffer (Invitrogen) at 150 V for 1 h.

Duplicate gels were run simultaneously and stained for total protein using Sypro Ruby (Invitrogen) according to the manufacturer's directions. A stain for bromoperoxidase activity was used as described in Suthiphongchai et al. (2008) with the following modifications. Gels run under denaturing conditions were soaked in the Tris-glycine buffer lacking SDS for 1 h, then placed in a PhR stain containing 100 mM MES (pH 7.0), 40 mM H_2O_2, 200 μM Na_2VO_4, 100 mM potassium bromide, and 2 mM PhR.

For protein sequencing, the gel was stained with Coomassie Brilliant Blue as recommended by the NuPAGE Technical Guide (Invitrogen), and the protein band corresponding to the active band observed with PhR on a duplicate gel was excised in 1 mm × 1 mm cubes, placed in MilliQ (Milli-Q Plus Ultrapure Water Purification System, Millipore, Billerica, MA, USA) water, and kept at 4°C until sequencing. Peptide sequencing was performed using LC/MS/MS as described in (McCormack et al. 1997) at the University of California San Diego (UCSD) Biomolecular/Proteomics Mass Spectrometry Facility.

Phylogeny. *Synechococcus* sp. CC9311 VBPO (YP_731869.1) was compared with the National Center for Biotechnology Information's (NCBI; Bethesda, MD, USA) nr protein database using blastp (Altschul et al. 1997). Amino acid sequences from the results with highest similarity were aligned in ClustalX (Chenna et al. 2003) and trimmed in SE-AL v.2.0 (Rambaut 1996) to a 183-amino-acid region (see Fig. S1 in the supplementary material) that includes active site residues known to be conserved between VBPO sequences (Winter and Moore 2009). A neighbor-joining analysis was performed (Fig. 3) from 100 bootstrap replicates using PHYLIP (Felsenstein 1989), and the resulting tree was viewed in Figtree (Rambaut 2008).

VBPO in Synechococcus. We tested closely related (clade I) strains of *Synechococcus* (sp. CC9617, WH8020, WH8016) for the presence of a bromoperoxidase gene. Degenerate PCR primers (Table 1; VDEGF, VDEGR) were designed based on residues conserved between cyanobacteria and rhodophyte VBPOs (see Fig. S1), to yield a 262 bp product for sync_2681 (CC9311 VBPO). Amplified products were cloned into the pcr4-TOPO vector (Invitrogen) as recommended by the manufacturer. Plasmids were isolated using a plasmid mini prep kit (Qiagen, Valencia, CA, USA) and sequenced using the T7 universal primer. For *Synechococcus* sp. WH8020, new primers (Table 1; VWholeF, VWholeR) were designed based on the 5′ and 3′ ends of sync_2681 in CC9311 to amplify the whole gene (1.8 kb), and the PCR product was sequenced. The active site and vanadium-coordinating residues, conserved between vanadium bromo-peroxidases and chloro-peroxidases, are annotated based on homology to those described in *Curvularia inaequalis* (Raugei and Carloni 2006) and *Ascophyllum nodosum* (Weyand et al. 1999).

Cyanobacterial VBPO in the environment. To test whether cyanobacterial-like VBPO genes were present in the coastal pelagic environment, samples were taken from the pier at Scripps Institution of Oceanography (La Jolla, CA, USA) on seven dates through 2006 (February, April, May, August, October) and 2007 (April, May). Half-liter water samples were filtered on a 0.2 μm Supor filter disk (Pall Life Sciences, Ann Arbor, MI, USA), and the DNA was extracted (Palenik et al. 2009, Tai 2009). Degenerate PCR primers were designed for a conserved region of cyanobacterial VBPO (Table 1; PierF, PierR). Touch-down PCR was the most successful method of amplification. Starting at 63°C, the annealing temperature was decreased by 1°C every cycle for 13 cycles, and then kept at 54°C for 27 cycles. A clone library was constructed from PCR products using pcr-4 Topo vector (Invitrogen). Multiple clones were sequenced from successful PCR amplifications.

Table 1. Primer sequences used in this study. Degenerate primer code is as follows: S, G or C; R, A or G; Y, C or T; N, any base.

Primer name	Sequence
VDEGF	5′-GCR TGN CCN GCN CCR TAN GMN GG -3′
VDEGR	5′-AAR GCN GTN CGN TAY CAR AAR TT -3′
VWholeF	5′-ATG ACA GAT CAA CGC AAA CTC AC-3′
VWholeR	5′-CTA TCG CTG ACC GAT GCG ACG-3′
PierF	5′-AAG GCS GTG CGY TAY CAR AAG TTC AA-3′
PierR	5′-GCN AGY TTR TTN AGY TCN CCY TC-3′

Two environmental metagenomic databases were also accessed for this study. Sequences were retrieved using blastp with VBPO from *Synechococcus* sp. CC9311 against the GOS database (Seshadri et al. 2007). *Synechococcus* VBPO from CC9311 was also used to look for sequences in *Synechococcus* metagenomes from water taken at the Scripps Pier in La Jolla, California (B. Palenik and I. T. Paulsen, unpublished). Nucleotide sequences from pier samples were aligned in ClustalX and trimmed to an overlapping region (see Fig. S2 in the supplementary material). A neighbor-joining analysis was used to construct a phylogenetic tree in PHYLIP with 100 bootstrap replicates. *Acaryochloris marina* VBPO (YP_001515553) was used as an outgroup.

RESULTS

Activity assays. Haloperoxidase activity was detected in *Synechococcus* sp. CC9311 crude, soluble, and membrane protein extracts using multiple assays. Specific haloperoxidase activities of *Synechococcus* proteins used with the MCD, ThB, and triiodide assays are shown in Table 2. When *Synechococcus* sp. CC9311 crude extracts were used with MCD, the V_{max} for bromide oxidation was 0.400 ± 0.046 μmol · min^{-1} · mg^{-1}. No detectable activity was observed in crude protein extracts using chloride as the halide for the MCD assay. Crude protein extracts from *Synechococcus* sp. WH8020, a strain closely related to *Synechococcus* sp. CC9311, had lower activity levels than those of *Synechococcus* sp. CC9311. No detectable activity was seen in crude, soluble, or membrane protein extracts from *Synechococcus* sp. WH8102 using bromide as the halide in the MCD or ThB assay. Furthermore, when the MCD assay is used with crude protein from *Synechococcus* sp. CC9311, <0.01% VBPO specific activity is observed when no vanadium incubation step is included (Table 2).

We used the ThB assay to compare Br^- and I^- as VBPO substrates in crude protein extracts. When a

TABLE 2. Vanadium-dependent bromoperoxidase specific activity found in *Synechococcus*.

Organism	Halide*	Specific activity ($\mu mol \cdot min^{-1} \cdot mg^{-1}$)	Assay
Synechococcus sp. CC9311	I	0.910 ± 0.110[a]	I_3^-
	[0.1 mM]	0.019 ± 0.001[a]	ThB
	Br	0.400 ± 0.046[a]	MCD
	Br	0.165 ± 0.003[a]	ThB
	Br	0.560 ± 0.085[b]	MCD
	Br	1.01[c]	MCD
	Br	0.002 ± 0.0003[d]	MCD
	Cl	ND[a]	MCD
	Cl	ND[a]	ThB
Synechococcus sp. WH8020	Br	0.074 ± 0.013[a]	MCD
Synechococcus sp. WH8102	Br	ND[a]	MCD

[a]Crude protein extracts used in assay.
[b]Soluble protein fraction from *Synechococcus* sp. CC9311.
[c]Membrane-protein fraction from *Synechococcus* sp. CC9311, $n = 1$.
[d]Crude protein extracts not preincubated with sodium orthovanadate.
*100 mM halide used unless otherwise indicated.
ND, not detected; MCD, monochlorodimedone; ThB, thymol blue.

Michaelis–Menten nonlinear regression was fit to the ThB assay results, VBPO in *Synechococcus* sp. CC9311 has a greater V_{max} with bromide (0.165 ± 0.005 $\mu mol \cdot min^{-1} \cdot mg^{-1}$) than with iodide (0.019 ± 0.001 $\mu mol \cdot min^{-1} \cdot mg^{-1}$). The half-saturation constant, K_m, was also larger for bromide (1.525 ± 0.184 mM) than iodide (0.0237 ± 0.003 mM). No activity was detected when chloride was used as the halide in the ThB assay.

The membrane protein fraction had 1.8 times greater specific activity than the soluble protein fraction (0.56 ± 0.085 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ and 1.01 $\mu mol \cdot min^{-1} \cdot mg^{-1}$, respectively) using the MCD assay.

SDS-PAGE and in-gel staining. In-gel staining for bromoperoxidase activity using PhR showed a single band of 220,000 Da in crude protein extracts (Fig. 1) as well as in soluble and membrane proteins extracted from *Synechococcus* sp. CC9311. The development of the band was dependent on the presence of both bromide and hydrogen peroxide (data not shown). Active bands appeared more slowly in the absence of vanadium, usually developing over 10 min without vanadium, while developing sooner when vanadium is present. No bands developed in lanes containing proteins from *Synechococcus* sp. WH8102.

The active band from the membrane fraction (see Materials and Methods) of *Synechococcus* sp. CC9311 was excised and sequenced using LC/MS/MS. A single protein was shown to be dominant, having 80% amino acid coverage from 51 peptides of the annotated VBPO (sync_2681). Though no other proteins were present in significant quantity or coverage, a ferritin (sync_1539) was present with three peptides providing 26% coverage of the gene.

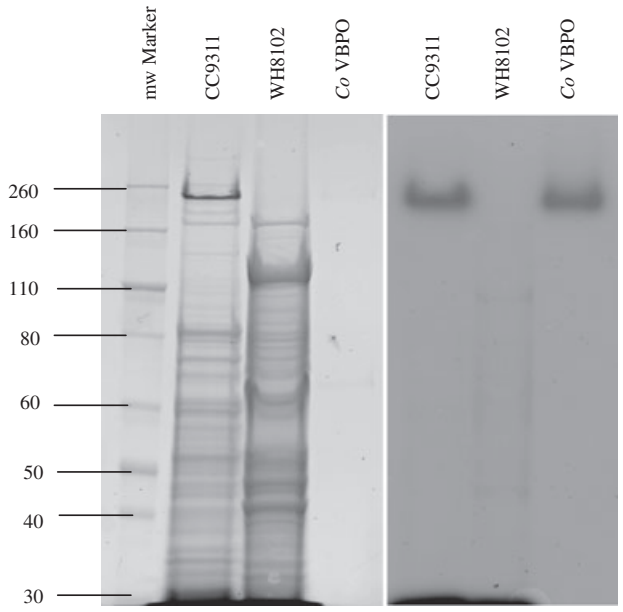

FIG. 1. SDS-PAGE gels of crude protein extracts from *Synechococcus* strains WH8102, CC9311, and vanadium-dependent bromoperoxidase (VBPO) from *Corallina officinialis* (*Co* VBPO, Sigma) stained with Sypro Ruby (left) and with phenol red (right) for bromoperoxidase activity. The migrations of molecular mass standards are indicated on the left in kDa.

VBPO in Synechococcus. Clade I *Synechococcus* strains for which genome data are not available (WH8020, CC9617, WH8016) were screened for VBPO genes using degenerate primers designed to amplify a short region of the bromoperoxidase gene. PCR amplification was only seen in CC9311 and WH8020. Subsequent sequencing of the entire VBPO gene found in *Synechococus* sp. WH8020 revealed 92.88% nucleotide identity and 95% amino acid identity with *Synechococcus* sp. CC9311, with no gaps (Fig. 2). These results correlate with *Synechococcus* sp. CC9311 whole-genome microarray studies using DNA–DNA hybridizations with these clade I strains, which showed a lack of VBPO gene hybridization in CC9617 and WH8016 (B. Palenik and I. T. Paulsen, unpublished).

Phylogeny. A phylogenetic analysis of vanadium bromoperoxidases and related haloperoxidases is shown in Figure 3. *Synechococcus* sp. CC9311 VBPO has the greatest amino acid identity to the two VBPO genes found in *Acaryochloris marina* MC11017 (53%, 35%), as well as the VBPO gene found in *Synechococcus* PCC7335 (46%). With respect to eukaryotic macroalgae, VBPO in *Synechococcus* sp. CC9311 has a greater similarity to rhodophyte bromoperoxidase (34% amino acid identity to *C. officinalis* 1 and 2) than to phaeophyte bromo- and iodo-peroxidases (18% identity to *L. digitata* B1 and B2; *L. digitata* I1 and I3). There are several cyanobacterial sequences annotated as type 2 phosphatidic acid phosphatases (PAP2)/haloperoxidases for which no enzyme

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CC9311   MTDQRKLTAQRVREDANALAAGRIHPRHQANGDEQRYESANYAMSFTKGLDHNTTTGLIE
WH8020   MTDQRKLTAQRVREDANALAAGRVQPQHQANGDEQRYESASYPMSFTKGLDHNTTTGLVE
                              :: :             . .               :

CC9311   QSGDFEAFRSAIDNGFAEDFTRHVAVPRAEPRRKWEAPTAGTVYELQGPDPQAVTIPPAP
WH8020   QSGDFEAFRSAIDNGFAEDFTRQVPVPHAEPRRKWEAPTAGTVYELQGPDPQAVTIPPAP
                              : .  :

CC9311   ALCSDELTFEMAEVYELALLRDLPFNAFVAGGGSAALADSTARLNSLAYAQDGFNRRPRT
WH8020   ALCSDELTFEIAEVYELALLRDLPFNAFVAGGGSAALADSTARLNNLAYAQDGFNSRPRK
                 :                                        .          .

CC9311   TNSSNQLDAQTVFRGSSPGVDQGPYLSQFMLIGNASPSEGITPEQGFINFGAQRIDQRVL
WH8020   TNSSKQLDAQTVFRGSSPGVERGPYLSQFMLIGNASPSEGITPDQGFINFGAQRIDQRVL
             :                ::                      :

CC9311   EARQQDDYMMKWDDWHRVQQGYEVRADRFDPCKSSGPGQAFTGQRRFIHTPRDLATYVHV
WH8020   EARQQDDYMMKWDDWHRVEQGYEVRGDRFDPCKSSGPGQAFTGQRRFIHTPRDLATYVHV
                           :      .

CC9311   DALYQAYLNACLLLLGNGTAFDPGFDLLSGGGEGLLHDPAGGQKVPLNAGGFALWGGPHV
WH8020   DALYQAYLNACLLLLGNGTPFDPGFDLLSGGGEGLLHDPASGQKVPLNAGGFALWGGPHV
                           .                        .
                      ------>
CC9311   LSLVTEVATRGLKAVRYQKFNNHLRLRPEALAARIEKAQEIESRFPTICGCFSEMASDLQ
WH8020   LSLVTEVATRGLKAVRYQKFNNHLRLRPEALAARIEKAQEIESRFPEICGCFSEMASDLQ
                                    ==========
CC9311   QVVDLIRNHNQSLAGEATALLPMAFAEGSPMHPAYGAGHATVAGACVTILKAFFNTSALF
WH8020   QTVDLIRNHNQSLAGDATALLPMAFAEGSPMHPAYGAGHATVAGACVTILKAFFNTSALF
          .              :

CC9311   VKINDVAGFHSKQHILARLKCGDSVEAGAYQETDCGKRLEFERCGSFHLIEGKYATFKPD
WH8020   VKINDLAGFYSKQHILDRLKCGDSVEAGAYQVTDCGKRLEFERCGSFHLIEGKDATFKPD
              :   :
                 <------
CC9311   GKTNQSCCPLTLEGELNKLAANISIGRNMAGVHYFSDYYDSLRMGEEIAIGILEEQALCY
WH8020   GTTNKSCCPLTLEGELNKLAANISIGRNMAGVHYFSDYYDSLRMGEEIAIGILEEQALCY
          .   :

CC9311   KTDPFVLSVPTFDGDVRRIGQR
WH8020   KTDPFVLSVPTFDGDVRRIGQR
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FIG. 2. Amino acid alignment of vanadium-dependent bromoperoxidase from *Synechococcus* sp. CC9311 and WH8020. Active site is shown by the solid bar, whereas vanadium-coordinating residues are shown in bold, as determined by homology to *Ascophyllum nodosom.* Arrows indicate residues used for the design of environmental probes (PierF and PierR). Periods indicate amino acid substitutions.

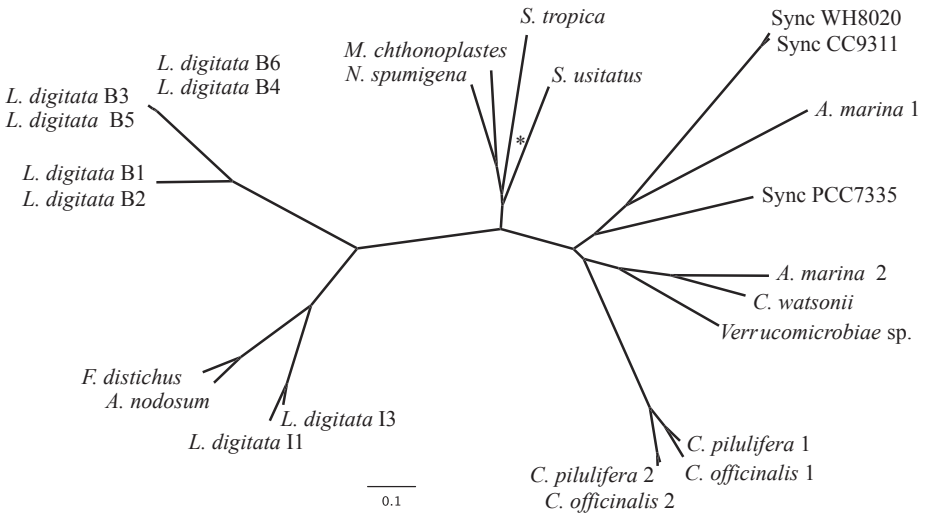

FIG. 3. Neighbor-joining tree of known bromoperoxidase sequences and related genes. Amino acid sequences obtained from the National Center of Biotechnology Information using *Synechococcus* sp. CC9311 (YP_731869.1) in a BLASTp. Asterix (*) indicates only node with <50% bootstrap support. Accession numbers are shown in Table S1 (see the supplementary material), and the alignment of select sequences is shown in Figure S1 (see the supplementary material).

activity has been reported (*Nodularia spumigena* CCY9414, *Microcoleus chtonoplastes* PCC7420, *Crocosphaera watsonii* WH8501, and *Synechococcus* sp. PCC7335). As these sequences encode the VBPO active site and could act as haloperoxidases, they were left in this analysis.

VBPO in the environment. To investigate the occurrence of VBPO in the coastal pelagic environment,

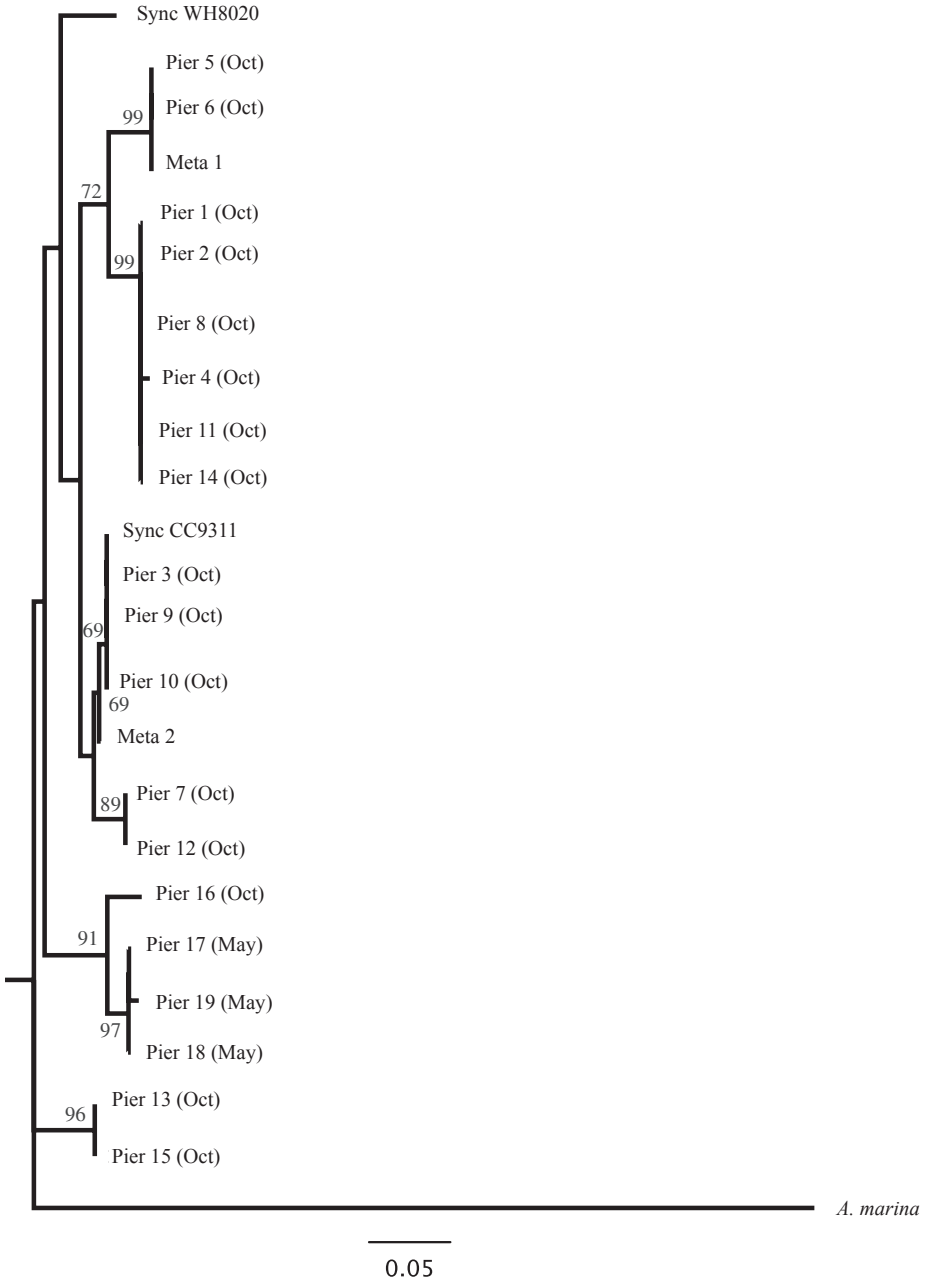

FIG. 4. Neighbor-joining tree of environmental vanadium-dependent bromoperoxidase sequences. Nucleotide sequences were trimmed to a conserved region of ~180 bp (alignment shown in Figure S2 (see the supplementary material). ''Pier #'' samples were obtained from a degenerate primer (PierF and PierR) clone library from the Scripps Pier in La Jolla, California. Samples labeled ''Meta'' were obtained from *Synechococcus*-specific metagenomes. Bootstrap values >50 shown as significant, from 100 replicates.

degenerate PCR primers were applied to environmental DNA samples from surface samples taken off the Scripps Pier (La Jolla, CA, USA), designed to amplify a conserved region of the VBPO gene, including the active site. Amplification was successful for the February, May, and October 2006 samples. Clones were obtained and sequenced from May (three total) and October of 2006 (16 total) and are shown in our analysis. When compared with known VBPO sequences, the clone sequences are closely related to *Synechococcus* sp. CC9311 VBPO (Fig. 4), with >87% nucleotide identified among all clones. Multiple clusters of sequences were observed from our clone library and local metagenomic data, with one cluster of sequences very closely related to VBPO from *Synechococcus* sp. CC9311 (98%–99.8% nucleotide identities to *Synechococcus* sp. CC9311 for Pier 3, 9, and 10 clones).

When a blastp search of the VBPO amino acid sequence from *Synechococcus* sp. CC9311 was performed against the GOS All Metagenomic ORFs (P) database (http://camera.calit2.net/) with a cutoff of E-06, 24 peptides were obtained representing 10 scaffolds. The scaffold sequences are from coastal, hypersaline lagoon, and mangrove sites. The top hit, JCVI_PEP_1105086041153 from GS033-Punta Cormorant, Floreana Island (Hypersaline Lagoon), Ecuador, is 55% identical to *Synechococcus* sp. CC9311 VBPO over a 300-amino-acid region. As this sequence and few of the scaffolds overlapped the

VBPO region obtained from PCR from the Scripps Pier, we did not include them in Figure 4. However, nine scaffolds appear to be from cyanobacteria, although none are demonstrably from clade I *Synechococcus*.

DISCUSSION

This is the first study to establish the presence of a functional VBPO in *Synechococcus*. Presence of this gene is not a genus-wide characteristic, as it is only found in some strains of clade I *Synechococcus* sp. (CC9311 and WH8020), but not found in other clade I strains tested (CC9617, WH8016), nor in representatives of other marine *Synechococcus* clades for which genome sequences are available, such as WH8102 (clade III), CC9902 (clade IV), or CC9605 (clade II). Homologous sequences are present in the genomes of *Acaryochloris marina, Synechococcus* sp. PCC7335, and *Crocosphaera watsonii*, suggesting the presence of functional bromoperoxidases in other marine or estuarine cyanobacteria.

Determining the distribution of VBPO among related organisms is important in exploring the link between red algal and cyanobacterial bromoperoxidases. In doing so, an important consideration is that VBPO (sync_2681) in *Synechococcus* sp. CC9311 appears to be in a cluster of horizontally transferred genes, encompassing a region of $\sim$ 29 kbp. These 32 genes, including a putative mechanosensitive ion channel (sync_2685), amidotransferanse (sync_2679), diguanylate cyclase (sync_2671), as well as hypothetical proteins, all lack homologues in 11 other marine *Synechococcus* with whole genomes sequenced (Dufresne et al. 2008). Furthermore, along with neighboring genes, VBPO is not represented in a coastal *Synechococcus* environmental metagenome, suggestive of a gene recently acquired by *Synechococcus* sp. CC9311 and closely related strains (Palenik et al. 2009). *A. marina* has one of the largest prokaryotic genomes, which includes two bromoperoxidase sequences that are the most similar known amino acid sequences to VBPO in *Synechococcus* sp. CC9311 (Swingley et al. 2008). *A. marina* is also a common epiphyte of red algae (Murakami et al. 2004) that have bromo-peroxidases and chloroperoxidases and produce a variety of halogenated metabolites. One possibility is that the bromoperoxidase gene was transferred from a red alga to an epiphytic cyanobacterium and subsequently spread to other photoautotrophic prokaryotes. This concept may be supported by the lack of ubiquity of the gene among *Synechococcus* strains. Alternately, the gene could have been acquired by a eukaryotic alga from an epiphytic prokaryote. However, more genomes and homologous gene sequences are necessary to support either lineage.

One example of a gene of eukaryotic origin acquired by cyanobacteria is the class I algal fructose bisphosphate aldolase (FBA) gene. This eukaryotic version of the FBA gene was recently found in several *Synechococcus* strains, including *Synechococcus* sp. CC9311 and CC9902 as well as multiple *Prochlorococcus* strains (Rogers et al. 2007). Cyanobacterial class I FBA genes cluster with the rhodophyte homologues of this plastid-targeted, metabolically important enzyme. Though the origin of VBPO in *Synechococcus* is not clear at this time, a similar evolutionary relationship may exist to rhodophyte VBPO homologues.

Enzyme activity. VBPO in *Synechoccus* sp. CC9311 is capable of oxidizing bromide and iodide but is not able to measurably oxidize chloride. Specific VBPO activities measured in this study are similar to previously reported VBPO activities (U = μmol MCD brominated $\cdot$ min^{-1}) measured in crude protein extracts from eukaryotic macroalgae, which include 3.2 U $\cdot$ mg^{-1} (Krenn et al. 1989) in *A. nodosum*, 0.14 U $\cdot$ mg^{-1} in the rhodophyte *Kappaphycus alvarezii* (Kamenarska et al. 2007), and 1.6 U $\cdot$ mg^{-1} in crude extracts from *Gracilaria* sp. (Suthiphongchai et al. 2008). VBPO from *Synechococcus* sp. CC9311 had a stronger affinity (lower K_m) toward iodide than bromide, though on the same order of magnitude as results found in *K. alverezii* (Kamenarska et al. 2007). Also, a greater specific activity was observed when triiodide accumulation was used to measure VBPO activity compared with using the thymol assay, which is a more direct comparison to the ability of VBPO to oxidize bromide.

Protein extracts from *Synechococcus* sp. WH8020 have similar specific activity toward bromide as *Synechococcus* sp. CC9311. However, *Synechococcus* sp. WH8102, which lacks a predicted bromoperoxidase gene in its genome, has no detectable bromoperoxidase activity when using MCD, ThB, or PhR as the organic substrate.

VBPO is active in both soluble and membrane-protein fractions in *Synechococcus* sp. CC9311. The soluble fraction maintains about half the specific activity of the membrane fraction, suggesting that this may primarily be a membrane-associated protein. Alternately, if the active enzyme is multimeric, this increase in mass may cause the protein to partially pellet during the ultracentrifugation, membrane protein isolation step used in this study. Bromoperoxidases have been shown to be outer-membrane bound as well as plastid-targeted enzymes in eukaryotic algae (Manley 2002).

SDS-PAGE analysis. The protein band that displayed VBPO activity ran at 220 kDa under both native and denaturing conditions, despite a predicted mass of 68 kDa for the product of sync_2681. The active band ran at the same mass as the bromoperoxidase from *C. officinalis*, which has a predicted MW of 64 kDa and is a homododecamer in situ (Coupe et al. 2007). VBPO is also known to form homodimers in brown algae (Weyand et al. 1999). An inactive band appeared $\sim$ 64 kDa under denaturing conditions in the *C. officinalis* VBPO

control. Bromoperoxidases are known to have high thermostability, only showing a gradual loss in activity at 80°C in *A. nodosum* (Krenn et al. 1989) and thus may not have been denatured after heating at 70°C for 10 min.

By sequencing the active band from SDS-PAGE gels stained with PhR, we have demonstrated that the only protein responsible is the single gene product of the annotated bromoperoxidase.

It will be important to determine if an organic molecule(s) is being halogenated in vivo before inferring a physiological function for VBPO in *Synechococcus* sp. CC9311. VBPO does not appear to be in a putative biosynthetic operon in *Synechococcus* sp. CC9311, as is the case for the vanadium-dependent haloperoxidase gene in two strains of *Streptomyces* (Winter et al. 2007), in which a vanadium chloroperoxidase is active as part of the biosynthetic pathway for napyridiomycin. This possibility does not preclude the interaction of VBPO with other gene products of *Synechococcus* sp. CC9311 but does not suggest an obvious organic substrate that is being brominated by the enzyme. Alternatively, the molecule may not originate from *Synechococcus* itself. For example, in pelagic ecosystems, dissolved organic carbon (DOC) has been implicated in providing the organic material that is brominated and found in diatoms (Hill and Manley 2006). An extracellular source, such as DOC, may be the substrate for pelagic *Synechococcus* VBPO.

The production of brominated organic compounds has been demonstrated in numerous microalgae including cyanobacteria. Symbiotic *Oscillatoria* sp. produce polybrominated diphenyl ethers (Unson et al. 1994, Agrawal and Bowden 2005). Other filamentous cyanobacteria, such as *Lyngbya,* are renowned for the production of halogenated natural products (Nogle and Gerwick 2003, Edwards et al. 2004). Interestingly, volatile halogenated organic compounds (VHOCs) have been associated with a mixed-cyanobacterial bloom in the Baltic sea (Karlsson et al. 2008), but no enzyme was tested as the source. VHOCs, particularly methyl halides, have also been suggested as by-products of the bromoperoxidase reaction in eukaryotic algae. Moore et al. (1996) demonstrated that multiple diatom cultures produce bromo- and iodo-methane while simultaneously finding haloperoxidase activity in these cultures. Brownell et al. (2010) reported that despite finding methyl halide production in cultures of both *Synechococcus* and *Prochlorococcus*, extrapolated production rates did not appear to contribute significantly to oceanic production rates of these molecules and conflicted with previously reported values. As the presence of VBPO in *Synechococcus* is strain specific, more work is needed to correlate methyl halide production to VBPO activity in cyanobacteria, particularly *Synechococcus*. Halomethane production has also been observed in several macroalgae. Further work should focus on determining the brominated organic products of VBPO in *Synechococcus*. Such information could lead to novel compounds or insights into substrate specificity.

Alternatively, the *Synechococcus* VBPO could contribute to the detoxification of photosynthetically produced hydrogen peroxide in vivo, as evidence has previously supported this role in eukaryotic macroalgae (Manley and Barbero 2001, and Ho et al. 2009). Future physiological studies will address this possibility as well. A better understanding of the function of VBPO may provide insights into ecological adaptations of *Synechococcus* as well as the roles of this enzyme in other organisms.

Bromoperoxidase in the environment. We found that *Synechococcus*-like bromoperoxidase genes are present and diverse in the coastal environment. After combining sequences from our clone library with those obtained from coastal *Synechococcus* metagenomes (Palenik et al. 2009, B. Palenik and I. Paulsen, unpublished), several clusters of sequences become apparent. However, all sequences were >87% identical. Metagenome sequences with 80% or higher sequence identity tile reliably to reference genomes (Tai 2009), suggesting that VBPO sequences with >87% identity are from clade I *Synechococcus* strains. However, we cannot rule out the possibility of recent HGT to other microbes. There was no correlation between the sampling date and the clustering of a sequence, suggesting that the VBPO genes detected in this study are in organisms that are found in May and October. Interestingly, VBPO was not detected on other sampling dates (April, August). All but one sequence from our library had conserved active site residues as predicted in previous literature (Carter et al. 2002), suggesting that these cyanobacterial-like VBPOs are functional in the pelagic environment.

Concluding remarks. This study brings light to the diversity of bromoperoxidase genes in free-living, unicellular cyanobacteria. We have demonstrated that *Synechococcus* sp. CC9311 encodes an active bromoperoxidase, the product of sync_2681. We have further observed an active bromoperoxidase gene in another clade I strain of *Synechococcus* as well as genetically related bromoperoxidase genes in coastal ocean samples.

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Supplementary Material

The following supplementary material is available for this article:

Figure S1. Alignment of the well-conserved region between cyanobacterial and eukaryotic algal vanadium-dependent bromoperoxidases (VBPOs) used to construct the phylogenetic tree shown in Figure 3. Sequences were obtained from the National Center for Biotechnology Information's nr protein database, and accession numbers can be found in Table S1 (see supplementary material). Arrows indicate regions used to design the primers VDEGF and VDEGR.

Figure S2. Nucleotide alignment of the Scripps Pier clone library to metagenomic sequences (see Materials and Methods). This alignment was used for the neighbor-joining analysis and phylogenetic tree construction shown in Figure 4.

Table S1. Gene codes and accession numbers of the sequences used in the neighbor-joining tree of vanadium-dependent bromoperoxidase (VBPO) genes shown in Figure 3.

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