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OBLIGATE BROMINATING ENZYMES UNDERLIE BROMOFORM PRODUCTION BY MARINE CYANOBACTERIA[1]

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Marine algae are prolific producers of bromoform ($CHBr_3$). This naturally produced molecule is a potent environmental pollutant as it volatilizes into the atmosphere and contributes to depletion of the ozone layer in a manner akin to, and in magnitude similar to, man-made chlorofluorocarbons. While phototrophs such as seaweeds, diatoms, and dinoflagellates are known sources of bromoform, additional as yet unknown biogenetic sources of bromoform exist in the oceans. Here, using halogenating enzymes as diagnostic genetic elements, we demonstrate that marine cyanobacteria also possess the enzymological potential for bromoform production. Using recombinantly purified vanadium-dependent bromoperoxidases from planktonic and bloom-forming marine cyanobacteria in *in vitro* biochemical assays, we reconstitute the enzymatic production of bromoform. We find cyanobacterial bromoform synthesizing enzymes to be obligate brominases possessing no chlorinating activities. These results expand the repertoire of marine biotic sources that introduce this pollutant in the atmosphere.

Key index words: **bromoform; cyanobacteria; haloperoxidases; ozone-depletion**

***Abbreviations*: VHPO, vanadium-dependent haloperoxidase; VBPO, vanadium-dependent bromoperoxidase; NOX, NAD(P)H oxidase; MBP, maltose binding protein**

The high concentration of halides in seawater makes the marine environment an important player in global halogen cycling. Of particular environmental relevance are the polybromomethane compounds ($CH_{4-n}Br_n$; n = 1–3) that are produced by biotic and abiotic processes in the ocean. These marine molecules volatilize into the atmosphere (Simpson et al. 2015). Although a diverse mixture of chlorinated, brominated, iodinated, and mixed halogenated methanes are detected in marine metabolomes, bromoform ($CHBr_3$) is the most abundant metabolite and is a major contributor to biogenic bromine released into the atmosphere (Sturges et al. 2000, Warwick et al. 2006, Tegtmeier et al. 2015). At the tropospheric boundary, bromoform reacts with and degrades the ozone layer. Contribution to ozone degradation by bromoform is substantial, up to 30% of ozone degradation can be attributed to bromoform that is naturally produced in the oceans (Salawitch 2006, Navarro et al. 2015).

Several photosynthetic marine organisms produce bromoform. Among these, seaweeds are exceptionally prolific producers (Paul and Pohnert 2011). Prior work has demonstrated that seaweeds use bromoform as a chemical defense against biofouling epiphytic bacteria (Paul et al. 2006). Building on prior reports which demonstrated the involvement of peroxidases in bromoform production in seaweeds (Theiler et al. 1978), recently, we determined the genetic and biochemical basis for bromoform production in the red macroalga *Asparagopsis taxiformis* (Thapa et al. 2020). Bromoform is also produced by diatoms and dinoflagellates (Tokarczyk and Moore 1994, Carpenter and Liss 2000). Though the ecological roles and biogenetic basis for bromoform production in phytoplankton remain unknown, the participation of peroxidase enzymes has been hinted (Moore et al. 1996). Recently, Palenik, Brahamsha, and coworkers demonstrated the production of bromoform by a marine coastal planktonic cyanobacterium and using in vivo gene deletion experiments, provided evidence for the participation of a peroxidase in bromoform biosynthesis (Johnson et al. 2011, 2015). Cyanobacteria found in the open ocean are also a source of bromoform; bromoform production has been observed from marine cyanobacterial blooms (Karlsson et al. 2008, Roy et al. 2011).

In abovementioned studies, bromoform production is tied to the activity of vanadium-dependent haloperoxidases (VHPOs; Fig. 1). The role of VHPOs in the production of halogenated molecules

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in seaweeds is well established (Carter-Franklin and Butler 2004, Wever et al. 2018). These enzymes are also involved in natural product biosynthesis in marine bacteria (Miles et al. 2017, McKinnie et al. 2018a). The biochemical activity of VHPOs involves hydrogen peroxide as a co-substrate; halides are oxidized to hypohalous acids ($X^- \rightarrow HOX$; X = Cl, Br, I) with hydrogen peroxide acting as the terminal electron acceptor via the covalently bound vanadate cofactor (Winter and Moore 2009, Agarwal et al. 2017; Fig. 1). Subsequent electrophilic substitutions by haleniums are substrate promiscuous, as was recently reported for the recombinant VHPO from marine cyanobacterium *Acaryochloris marina* (*Am*VHPO) which was shown to chlorinate and brominate a range of aromatic substrates (Frank et al. 2016). With the notable exception of marine sediment-derived actinobacteria (Winter and Moore 2009, McKinnie et al. 2018b), the preponderance of phototrophs in utilizing VHPOs for the production of bromoform and other halogenated natural products is perhaps tied to hydrogen peroxide production by the overenergization of the photosynthetic electron transport chain (Pospíšil 2016, Mullineaux et al. 2018). In addition, dedicated oxidases that transfer electrons from NAD(P)H to molecular oxygen, thus called NAD(P)H oxidases (NOXs), also generate hydrogen peroxide (Fig. 1). The biochemical activity and genetic colocalization of a NOX encoding gene with bromoform-producing VHPO encoding genes in seaweeds was recently demonstrated (Thapa et al. 2020).

In this report, we provide in vitro reconstitution of bromoform production by VHPOs from marine cyanobacteria. For biochemical characterization, VHPOs from two marine cyanobacterial genera *Synechococcus* and *Trichodesmium* were chosen. *Synechococcus* are abundant in both coastal and open ocean ecosystems and are major contributors to photosynthetic carbon fixation in the oceans (Flombaum et al. 2013). *Trichodesmium*, bloom-forming filamentous cyanobacteria, are abundant in both tropical and subtropical oceans and are primary players in the global nitrogen cycle (Bergman et al. 2013, Capone et al. 1997). Specifically, the biochemical activities of recombinantly expressed VHPOs from coastal planktonic *Synechococcus* sp. CC9311 and from bloom-forming *Trichodesmium erythraeum* were queried. We demonstrate that these VHPOs are obligate bromoperoxidases, thus henceforth referred to as vanadium-dependent bromoperoxidases (VBPOs; *Ss*VBPO and *Te*VBPO) and that they efficiently catalyze the production of bromoform from hydrocarbon substrates that resemble fatty acid biosynthetic intermediates. While bromoform production from *Synechococcus* has prior precedent (Johnson et al. 2011, 2015), *T. erythraeum* is not a previously known bromoform producer. Our study thus establishes the validity of using VHPO gene sequences in marine cyanobacterial genomes as a marker for bromoform biosynthetic potential. In the postgenomic age, such gene-to-function insights can be used to guide the discovery of additional biotic species that have potential detrimental effects on the environment.

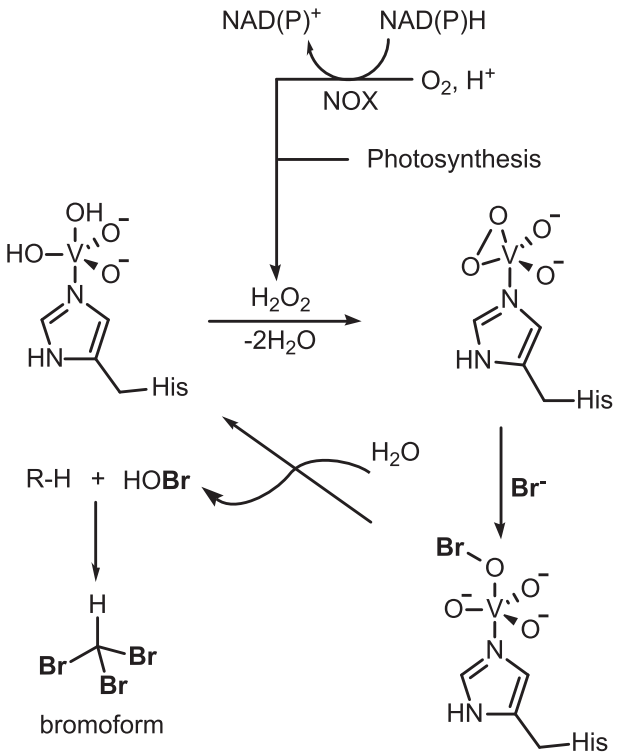

FIG. 1. Participation of VHPOs in bromoform biosynthesis. The vanadate cofactor is covalently bound to an enzyme active site histidine residue side chain. Hydrogen peroxide; produced by NOX enzymes and as a byproduct of photosynthetic electron transport chains oxidizes the cofactor to peroxovanadate intermediate which undergoes halide addition (bromide is illustrated here). Hydrolytic resolution then furnishes the hypobromous acid (HOBr) which serves as the effective halogenating agent. VHPOs that catalyze bromination, but not chlorination, are called VBPOs.

MATERIALS AND METHODS

Phylogenetic analysis. Full-length amino acid sequences of 46 VHPO proteins from different organisms were used for phylogenetic analysis (Table S1 in the Supporting Information). Multiple sequence alignment of VHPO proteins was generated using ClustalW (Larkin et al. 2007) alignment feature of MEGA 7 (Kumar et al. 2016). Protein sequence alignment thus generated was used to construct phylogenetic tree using default parameters of Maximum Likelihood method based on the Jones-Taylor-Thornton (JTT) matrix model (Jones et al. 1992). Initial tree for the heuristic search was generated using Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model followed by selection of topology with superior likelihood value. The tree with the highest log likelihood is shown in Figure 2.

Cloning, protein expression, and purification. Amino acid sequences for *Ss*VBPO and *Te*VBPO were used for synthesis of gene fragments optimized for expression in *Escherichia coli* (Twist Biosciences). A single adenosine nucleotide was added

to each synthetic DNA fragments using GoTaq polymerase (Promega) and then DNA fragments cloned into pGEM-T Easy vector (Promega) following manufacturer's instructions. The pGEM-T plasmid constructs for each gene fragment were verified by Sanger sequencing and then used as templates for PCR for cloning genes into *E. coli* expression vector. PCR reactions contained 20 ng template DNA, 0.4 µM primers, 2.5 mM dNTPs, 1X Phusion buffer, and 0.5 U Phusion DNA polymerase (ThermoFisher) in a 25 µL reaction. PCR cycling conditions were initial denaturation at 98°C for 2 min, 33 cycles of 98°C for 30 s, 55°C for 30 s, 72°C for 60 s, and final extension at 72°C for 10 min. PCR amplicons were purified using DNA Clean and Concentrator Kit (Zymo Research) and then cloned into pET28MBP (Lee et al. 2013) expression vector using NEBuilder HiFi DNA Assembly master mix following the protocols described by manufacturer (New England BioLabs).

Expression constructs, *Ss*VBPO/pET28MBP and *Te*VBPO/pET28MBP, were designed to add N-terminal His_6-MBP fusion tags to increase the expression and solubility of recombinant proteins. Gene constructs were transformed into *E. coli* BL21-Gold (DE3) cells (Agilent Technologies) for recombinant protein expression. A 25 mL starter culture was grown overnight (14-16 h) at 37°C with 180 rpm shaking using Luria-Bertani (LB) broth. 10 mL of this overnight culture was used for inoculation of 1 L terrific broth (TB) media containing kanamycin at a concentration of 50 mg · L^{-1}. Cultures were grown in 2.8 L baffle flasks at 30°C with shaking at 180 rpm until the OD_{600} reached 0.8. Cultures were then cooled by incubation for 1 h at 18°C and protein expression induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.3 mM. Cultures were grown for another 18 h at 18°C. Cultures were harvested by centrifugation, and cell pellet from 2 L culture was used for protein purification. All steps of protein purification were done on ice or at 4°C. Cells were resuspended in 80 mL buffer A (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 1 mM EDTA), cells lysed by sonication (15 s on/ 45 s off at a power of 40%) for 30 min and then centrifuged at 30,000*g* for 40 min. The supernatant was loaded on a 5 mL His-Trap Ni-NTA column (GE Healthcare) at flow rate of 1 mL/min using AKTA Prime FPLC system (GE Healthcare), column washed with 80 mL of buffer B (20 mM Tris-HCl (pH 8.0), 500 mM NaCl, 30 mM imidazole) at a flow rate of 2 mL · min^{-1} and protein eluted using 40 mL linear gradient to 100% buffer C (20 mM Tris-HCl, pH 8.0, 1 M NaCl, 300 mM imidazole). Eluant fractions were analyzed by gel electrophoresis, and fractions containing protein of interest were pooled. Protein samples were concentrated to 2.5 mL using Amicon centrifugal filters, loaded on a PD-10 desalting column (GE Healthcare), and eluted with 3.5 mL storage buffer (20 mM HEPES-Na, pH 7.5, 50 mM KCl, 10% v/v glycerol). Protein concentration was determined was using the Bradford assay, purified protein samples concentrated to desired concentration, and stored in 50 µL aliquots at −80°C. Fresh aliquots of *Ss*VBPO and *Te*VBPO recombinant proteins were used for enzyme assays.

Monochlorodimedone-halogenation (MCD) assay. Using previously described methods, the monochlorodimedone-halogenation assay was used to characterize the halide specificity of *Ss*VBPO and *Te*VBPO enzymes (Wever et al. 2018). Enzyme assays were conducted in a quartz cuvette at room temperature (~ 25°C), reaction volume 1 mL, and contained 100 mM Na-citrate (pH 6.5), 100 mM KBr or KCl, 100 µM monochlorodimedone (**1;** Fig. 3A), 10 µM sodium orthovanadate, 2 mM hydrogen peroxide, and 100 nM of *Ss*VBPO or 200 nM of *Te*VBPO. Reactions were initiated by enzyme addition and the absorbance recorded at 290 nm every 30 s for

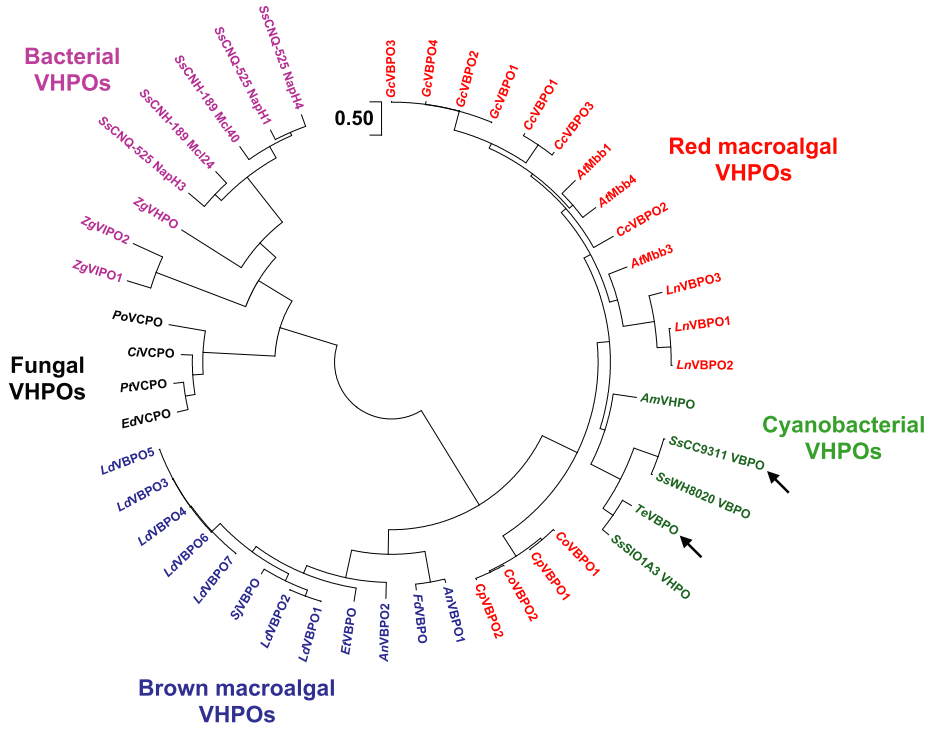

FIG. 2. Phylogenetic tree of VHPO protein sequences from different organisms. The phylogenetic tree is drawn to scale with branch lengths measured as the number of substitutions per site. Two cyanobacterial VHPOs shown by arrow were chosen for biochemical characterization.

15 min using NanoDrop OneC UV-Vis spectrophotometer (ThermoFisher). Triplicate assays for each halide were done for both enzymes.

Enzymatic bromoform biosynthesis. Enzyme assays for bromoform production were conducted as described before (Thapa et al. 2020). Reactions were initiated by adding 1 μL of 1 M hydrogen peroxide stock solution to a 1 mL reaction containing 100 mM HEPES-Na (pH 7.6), 50 mM KBr, 10 μM sodium orthovanadate, 1 mM substrate (pentane-2,4-dione (**2**; Fig. 4A) or heptane-2,4,6-trione (**3**)), and 1 μM *Ss*VBPO or 5 μM of *Te*VBPO enzymes. Hydrogen peroxide was continuously supplied during the reaction by the addition of 1 μL of 1 M stock solution every 10 min. After incubation of reactions at 30°C for 90 min, samples were transferred to 4 mL glass vials and reactions quenched by addition of 1 mL gas chromatography (GC)-grade diethyl ether and 100 μL brine. Samples were extracted by vigorous agitation using a vortex mixer, centrifuged at 1,500*g* for 10 min, and the top organic layer was withdrawn. The extraction process was repeated, organic layers pooled, and concentrated to 100 μL volume in vacuo. 2 μL of this extract was analyzed by gas chromatography-mass spectrometry (GC-MS). GC-MS analysis was performed in electron ionization (70 eV) mode using a DB-5ms ultra inert GC column (30 m length, 0.25 mm width, 0.5 μM film thickness; Agilent Technologies). Helium was used as the carrier gas (0.9 mL/min). Oven temperature was held at was 40°C for 3 min, increased to 200°C at a rate of 10°C · min^{-1} and held for 1 min with total run time of 20 min. Temperatures of injection port, interface, and ion source were 250°C, 300°C, and 230°C, respectively.

RESULTS

Phylogeny of VHPO proteins. Marine macroalgae are closely associated with cyanobacteria; cyanobacterial origin of macroalgal genes and macroalgal photosynthetic pigments is established (Murakami et al. 2004, Lee et al. 2018, Friedrich 2019). To discern the relationship, in any, between cyanobacterial and macroalgal VHPOs, phylogenetic analysis was conducted using 46 VHPO protein sequences from different organisms. The phylogenetic tree shown in Figure 2 was constructed using the Maximum Likelihood method and demonstrates that cyanobacterial VHPOs cluster with red seaweed VHPOs with a relatively greater phylogenetic distance from brown seaweed VHPOs. Of note, two of the red macroalgae represented here (*At*: *Asparagopsis taxiformis* and *Cc*: *Chondrus crispus*) are validated bromoform producers (Carpenter and Liss 2000). When compared to other organisms, cyanobacterial VHPOs remain distant from fungal and other bacterial VHPOs; here, bacterial VHPOs include marine actinobacterial VHPOs for which biochemical activities have been established (Winter and Moore 2009, Bernhardt et al. 2011, Miles et al. 2017, McKinnie et al. 2018b). Illustratively, amino acid identity of *Te*VBPO (from cyanobacterium *Trichodesmium erythraeum*; enzyme biochemically interrogated herein) to *At*Mbb1 (red macroalgae), *An*VBPO2 (brown macroalgae), *Ss*CNQ-525_NapH1 (marine actinobacteria), and *Ci*VCPO (fungal) is 47.97%, 24.67%, 19.25%, and 16.89%, respectively. Expectedly, brown and red macroalgal VHPOs formed their respective clades while sharing a common origination node. Similarly, marine bacterial and terrestrial fungal VHPOs also formed their distinct clades with no clustering of VHPOs observed between actinobacteria and bacteroidetes.

Functional characterization of cyanobacterial VBPOs. Amino acid sequence alignment of cyanobacterial *Ss*VBPO and *Te*VBPO with macroalgal VHPOs demonstrated the conservation of residues involved in binding the vanadate cofactor (Fig. S1 in the Supporting Information). Here, key residues include the histidine which provides the imidazole side chain for covalent attachment of the vanadate cofactor (Fig. 1), and residues that provide basic side chains to bind to the negatively charged vanadate equatorial and axial oxygen atoms. With this

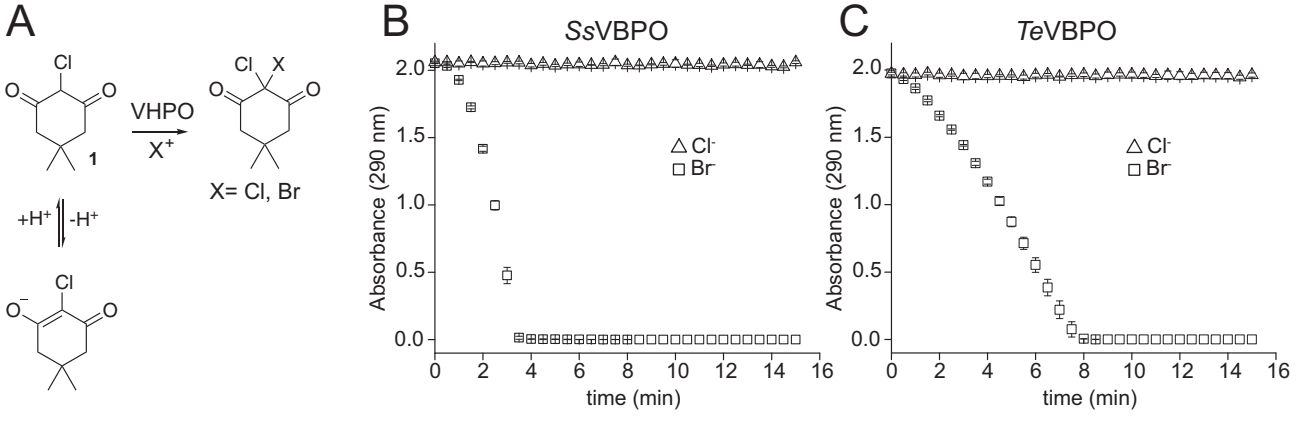

FIG. 3. Biochemical characterization of cyanobacterial VBPOs. (A) Halogenation of **1** offers a spectroscopic for monitoring halogenating activity of VHPO enzyme. Enzyme assays with (B) *Ss*VBPO and (C) *Te*VBPO using **1** as substrate, and KCl (open triangles) and KBr (open squares) as halide sources. Change in absorbance of substrate **1** at 290 nm was recorded every 30 s over 15 min. Assays were performed in triplicate, and data (mean and standard deviation) are plotted for each halide substrate.

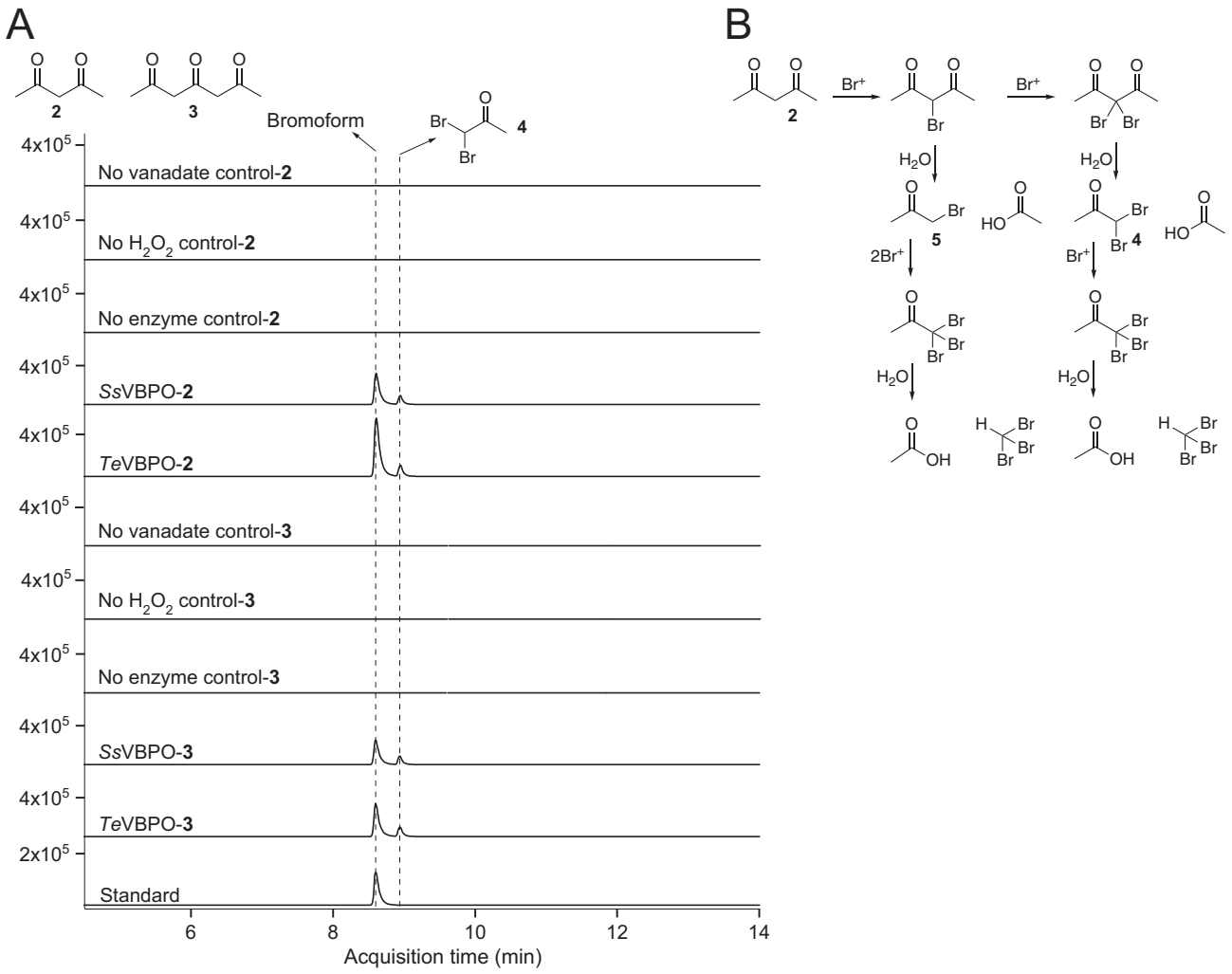

FIG. 4. Bromoform synthesis by cyanobacterial VBPOs. (A) GC-MS chromatograms showing in vitro production of bromoform and **4** using substrates **2** and **3** by purified recombinant *Ss*VBPO and *Te*VBPO enzymes. Control reaction that omit vanadate and hydrogen peroxide (H_2O_2) were conducted with *Ss*VBPO. (B) Reaction scheme for the production of bromoform and bromoacetones **4** and **5** by cyanobacterial VBPOs.

confirmation in hand, next, we proceeded to generate recombinant *Ss*VBPO and *Te*VBPO enzymes by heterologous expression in *E. coli*. N-terminal and C-terminal hexa-histidine tags did not yield recombinant proteins in a soluble form. Recombinant *Ss*VBPO and *Te*VBPO could be produced only when fused to a N-terminal maltose-binding protein (MBP). As a fusion tag, MBP improved the expression and resulted in soluble proteins, which still underwent degradative proteolysis in solution (Fig. S2 in the Supporting Information). Contrary to marine actinobacterial VHPOs, difficulty in heterologous expression and purification is often encountered for algal VHPOs with brown macroalgal VHPOs being especially difficult to produce recombinantly. Purified *Ss*VBPO and *Te*VBPO proteins were used for biochemical assays using **1** as a substrate. Halogenation of **1** quenches its absorbance thus providing an absorbance-based spectroscopic readout at 290 nm (Fig. 3A).

The monochlorodimedone-halogenation assay established both *Ss*VBPO and *Te*VBPO as obligate bromoperoxidases. Decrease in absorbance for **1** was observed only when bromide was present; no decrease in absorbance of **1** was observed with chloride (Fig. 3,B and C). The *Ss*VBPO was more proficient as compared to *Te*VBPO; a thousand-fold molar excess **1** was consumed by *Ss*VBPO in half the time as for *Te*VBPO with a greater initial rate of reaction (specific activity of *Ss*VBPO 47.6 U/mg, *Te*VBPO 10.2 U · mg^{-1}; Colin et al. 2003). The halide specificities of *Ss*VBPO and *Te*VBPO are consistent with bromoform producing macroalgal *Asparagopsis taxiformis* and *Chondrus crispus* VHPOs (Thapa et al. 2020). However, the obligate brominating activity of *Ss*VBPO and *Te*VBPO is in contrast

with the only other marine cyanobacterial VHPO which was experimentally established to be a chlorinase (Frank et al. 2016); halogenating enzymes are named per the most electronegative halide that they oxidize (Blasiak and Drennan 2009). To the best of our knowledge, *Ss*VBPO and *Te*VBPO are the as yet only known bacterial VBPOs; all prior VBPOs are derived from marine macroalgae. It should be noted that an erroneous assignment of halide specificity by the monochlorodimedone-halogenation assay alone is possible (Bernhardt et al. 2011). However, further biochemical characterization supports the halide specificity assignment (vide infra). Mechanistic determinants for halide specificity remain unknown (Agarwal et al. 2017).

Bromoform production by cyanobacterial VBPOs. Next, we queried the abilities of *Ss*VBPO and *Te*VBPO to produce bromoform in vitro. Bromoform producing VBPO enzymes from red macroalgae utilized β,δ-polyones **2** and **3** as substrates for bromoform synthesis. Since *Ss*VBPO and *Te*VBPO show high level of amino acid sequence identity and have halide specificities similar to bromoform producing macroalgal VBPOs, we assayed their enzymatic activities with **2** and **3**. Assays were conducted using purified proteins as catalysts and organic extracts of these assays were analyzed by GC-MS to identify the reaction products. Negative control reactions without addition of enzyme were also conducted for each substrate tested.

Both *Ss*VBPO and *Te*VBPO could use **2** and **3** as substrates to produce bromoform in in vitro reactions (Fig. 4A). Product identity was confirmed by comparison of mass spectra and retention times to a synthetic standard of bromoform.

In addition to bromoform, 1,1 dibromoacetone (**4**) and bromoacetone (**5**) were also observed as reaction products of the bromoform synthesis assay (Figs 4A, 5). Standards for **4** and **5** were not available; however, their identities can be dereplicated by annotation of the mass spectral fragmentation patterns (Figs. S3 and S4 in the Supporting Information). **4** and **5** were also observed as products generated in vitro bromoform production reactions by macroalgal VBPOs (Thapa et al. 2020). These bromoacetones have not been reported as cyanobacterial natural products though they have been observed in extracts of *Asparagopsis* seaweeds (McConnell and Fenical 1977). The production of **4** and **5** supports an halenium electrophilic substitution reaction mechanism, akin to the haloform reaction. Here, the most acidic γ-protons will be replaced by bromine atom(s) followed by hydrolytic cleavage of the C-C bond to furnish the bromoacetone and a carboxylic acid product (Fig. 4B).

The biosynthetic mechanism for production of monohalomethanes, such as CH_3Br, is different from that of polyhalomethanes (Agarwal et al. 2017); it was thus not surprising that we did not detect the in vitro production of CH_3Br by either *Ss*VBPO or *Te*VBPO. Crucially, we did not detect the production of CH_2Br_2 either. Both CH_3Br and CH_2Br_2 are reported to be produced by the *Synechococcus* strain from which *Ss*VBPO is derived though bromoform dominates the volatile brominated metabolome (Johnson et al. 2015). It is thus likely that more than one brominating activity exists within this cyanobacterium. Crucially, even though chloride was present in the in vitro assays described here, no chlorinated or mixed halogenated products were observed (Fig. S5 in the Supporting Information). This observation supports the halide specificity determined by the monochlorodimedone-halogenation assay. In addition to CH_3Br, the *Synechococcus* strain is also reported to produce chlorinated methanes; all three CH_3Cl, CH_2Cl_2, and $CHCl_3$ are produced in amounts exceeding that of the (poly)bromomethanes (Johnson et al. 2015). It is thus plausible that this cyanobacterium harbors multiple halogenating enzymes with differing halide specificities and product profiles.

DISCUSSION

Prior work from Palenik and Brahamsha had identified *Ss*VBPO to be involved in the biosynthesis of bromoform in *Synechococcus* (Johnson et al. 2011, 2015). However, the biochemical activity of the enzyme was not reported, specifically as it relates to halide specificity. Given that this cyanobacterial strain was found to produce other chlorinated, brominated, and iodinated methanes, clarification for the role of this enzyme in halomethane production was required. Here, we demonstrate that with the addition of a biogenic source of hydrogen peroxide, the *Ss*VBPO is sufficient, and specific for the production of bromoform. Bromoform production by *Trichodesmium erythraeum* has not been reported. However, the characterization of *Te*VBPO establishes that the biosynthetic potential for bromoform production exists within this filamentous marine cyanobacterium which is known to produce large blooms. While *Trichodesmium* is a prolific producer of natural products, including chlorinated hybrid peptides and polyketides, this is the first demonstration of brominating enzymology from this genus (Sudek et al. 2006, Via et al. 2018, McManus et al. 2020). A homology search reveals that marine filamentous bacteria of genera *Symploca*, *Leptolyngbya*, *Okeania*, and *Moorea*, among others, all possess VHPOs with as yet unidentified halide specificities and physiological roles. While the activities of these VHPOs are not yet established, it is likely that the genetic potential for bromoform biosynthesis is widely spread among marine cyanobacteria. In addition to diatoms and dinoflagellates, marine cyanobacteria could be underappreciated but important natural sources of oceanic bromoform. The ability of *Ss*VBPO and *Te*VBPO to use substrates **2** and **3**, as demonstrated previously for red

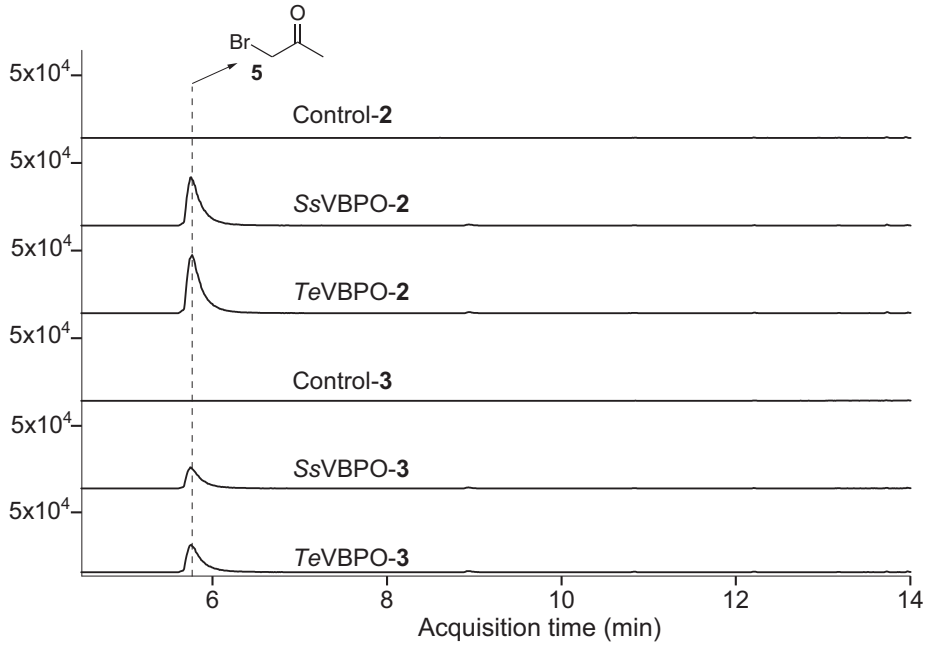

FIG. 5. Bromoacetone synthesis by cyanobacterial VBPOs. GC-MS chromatograms showing in vitro production of **5** in assays conducted with purified enzymes *Ss*VBPO and *Te*VBPO, and substrates **2** and **3**. Control reaction omits enzyme in the assay. Data shown are representative example from three independent experiments.

macroalgal VBPOs, also suggests a possibility that bromoform biosynthesis pathway may overlap between cyanobacteria and red macroalgae. **2** and **3** are surrogates for thiotemplated β-keto fatty acid biosynthetic intermediates, a primary biochemical process that is preserved in all forms of life. While physiological substrates for bromoform biosynthesis may be numerous and varied, a successful reconstitution of bromoform biosynthesis from **2** and **3** establishes that cyanobacteria do possess the requisite enzymology for bromoform production.

Phylogenetic relatedness, high level of amino acid sequence identity, and biochemical similarities between VBPOs from marine cyanobacteria and red macroalgae support the hypothesis that cyanobacterial VBPOs may share an evolutionary and functional relationship with red macroalgal VBPOs, perhaps through epiphytic or endosymbiotic relationships. On the other hand, the phylogenetic and biochemical differences between marine cyanobacterial and marine actinobacterial VHPOs are instructive to observe. Over the last decade, Moore has provided a detailed insight into the strict substrate specificities and intricate involvement of marine actinobacterial VHPOs in natural product biosynthesis (Diethelm et al. 2014, Miles et al. 2017, McKinnie et al. 2018b). The halide specificities of marine actinobacterial enzymes are different; they are all chlorinases. Furthermore, actinobacterial VHPOs colocalize with other natural product biosynthetic genes (Diethelm et al. 2014, Miles et al. 2017, McKinnie et al. 2018a,b). No such colocalization of cyanobacterial VHPOs with discernable natural product biosynthetic genes is observed (Fig. S6 in the Supporting Information). Cyanobacterial VHPO encoding genes are also not colocalized with genes that encode enzymes that could furnish H_2O_2 or β-carbonyl hydrocarbon substrates. The physiological roles of VHPOs in marine cyanobacteria thus seem to be different from that in actinobacteria. Given the demonstration that bromoform offers macroalgae protection against epiphytic colonization (Paul et al. 2006), it is possible that marine cyanobacteria may be utilizing a similar chemical defense strategy. This would be especially relevant for bloom-forming cyanobacteria such as *Trichodesmium erythraeum*.

Already, we know that a significant fraction, up to 30%, of molecular oxygen in the oceans is in the form of reactive oxygen species such as hydrogen peroxide (Hansel and Diaz 2020, Sutherland et al. 2020). Biogenetic sources that convert hydrogen peroxide to bromoform, such as VBPOs, can impact the global halogen cycling. As marine ecosystem is rapidly changing in response to increasing eutrophication and rising CO_2 levels, large phytoplankton blooms are becoming more common. How will these environmental changes contribute to bromoform production in the oceans? Is bromoform production limited only to phototrophic organisms? Can VHPO sequences serve as biomarkers to identify as yet elusive sources of bromoform in our oceans? As large-scale marine metagenomic data are

available and are expanding, future studies could query VHPO sequences in these data and test their biosynthetic potential for bromoform production. Activities aimed at mitigating the deleterious effect of these pollutants on the protective ozone layer will greatly benefit from a thorough inventory of the biogenic sources for such pollutants.

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AUTHOR CONTRIBUTIONS

H.R.T. and V.A. designed the research; H.R.T. performed the research; H.R.T. and V.A. analyzed data and wrote the paper.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web site:

Figure S1. Amino acid sequence alignment of VHPO proteins from red macroalgae and cyanobacteria.

Figure S2. SDS-PAGE analysis of recombinant VBPO proteins with His-MBP tag at the N-terminus: His-MBP-*Ss*VBPO (113.9 kDa) and His-MBP-*Te*VBPO (111.6 kDa).

Figure S3. Mass spectrum dibromoacetone (**4**) with arrow corresponding to its monoisotopic ion.

Figure S4. Mass spectrum of bromoacetone (**5**). Arrow corresponds to monoisotopic ion of bromoacetone.

Figure S5. Extracted ion chromatograms (EICs) for chlorinated methane products for in vitro assays performed using SsVBPO and TeVBPO for substrates (**A**) **2** and (**B**) **3** together with standards for dichloromethane and chloroform.

Figure S6. Gene neighborhoods for cyanobacterial VHPO encoding genes.

Table S1. List of GeneBank accession numbers and species information for VHPO sequences used in phylogenetic analysis in Figure 2