2004

A non-heme bromoperoxidase from an acorn worm slime-associated bacterium

Jasmine Kaur
San Jose State University

A NON-HEME BROMOPEROXIDASE FROM AN ACORN WORM SLIME-ASSOCIATED BACTERIUM

A Thesis

Presented to

The Faculty of the Department of Biological Sciences

San Jose State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science – Biological Sciences,

Molecular Biology and Microbiology

By

Jasmine Kaur

May 2004

APPROVED FOR THE DEPARTMENT OF

BIOLOGICAL SCIENCES

Sabine Rech

Dr. Sabine Rech

Patricia L Grilione

Dr. Patricia Grilione

Leslee A. Parr

Dr. Leslee Parr

APPROVED FOR THE UNIVERSITY

Pamela C Stark

ABSTRACT

A NON-HEME BROMOPEROXIDASE FROM AN ACORN WORM SLIME-ASSOCIATED BACTERIUM

by Jasmine Kaur

Bromoperoxidases are halogenating enzymes that catalyze the bromination of substrates in the presence of bromine and hydrogen peroxide. We have isolated a Gram positive bacterium (isolate # 4) from the slime associated with marine red-banded acorn worms, which exhibits bromoperoxidase activity. A total of fifty different bacteria were isolated, collectively, from slime dilutions and acorn worm body impressions. This strain was selected because of its characteristic iodine-like odor. The bacterium is an oxidase positive spore forming motile rod. The ribosomal DNA sequence shows 99% homology with *Bacillus cereus*. Growth studies showed that the strain exhibits bromination activity from approximately 10 hours to 28 hours of growth. The bromoperoxidase protein was purified from the isolate using ion exchange chromatography and gel filtration. The protein was purified to 95% homogeneity. An activity stain using a native PAGE gel and the phenol red assay shows the protein to be approximately 62kDa in size.

ACKNOWLEDGEMENTS

I would like to thank Dr. Rech for her patience, her understanding, her willingness to help, and for teaching me all about protein purification. I am thankful to Dr. Murray for his advice on numerous occasions, to Dr. Grilione for her support and the starting of this project, and to Dr. Okuda for his help and for providing me the monochlorodimedone (MCD) assay reagent. I would also like to thank Dr. Parr for reading and approving my thesis on short notice.

I would also like to thank my parents, sister, husband, and friends who now know more about bromoperoxidases than they ever wanted to know.

TABLE OF CONTENTS

PAGE

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

The biological importance of halogenated products ranges from defense mechanism chemicals for organisms to therapeutic drugs with anti-inflammatory or antibiotic activities [8]. Naturally occurring halometabolites include antibiotics (chlorotetracycline, chloramphenicol, griseofulvin, and pyrrolnitrin); antimicrobial agents (phenols, avrainvilleol, methyl alkyl, and alkyl vinyl ketones); anti-inflammables (griseofulvin); antifeedants (avrainvilleol) and many others [60].

Haloperoxidases are one group of enzymes responsible for halogenation. Halogenation is defined as the process of addition of a halogen (chlorine, bromine, fluorine, or iodine) to a compound by an enzymatic reaction. Haloperoxidases catalyze the oxidation of halide ions followed by organic substrate halogenation in the presence of hydrogen peroxide [61, 68, 69]. The halide ion (X^-) in the reaction below is chloride, bromide or iodide. Hydrogen peroxide reacts with the resulting electrophilic halide, which then reacts with organic substrates [61].

$$X^- + H+ + H_2O_2 + R_2CH \longrightarrow R_2CX + 2H_2O_2 \qquad [10]$$

Haloperoxidases are divided into three groups: iodoperoxidases, chloroperoxidases, and bromoperoxidases, based upon their halide specificity [10]. There are three types of haloperoxidase enzymes: the heme type, the bacterial non-heme type, and the eukaryotic non-heme type [10]. The heme-type haloperoxidases have a protoporphyrin IX as their prosthetic group [32, 90] and are inhibited by azide [10]. The bacterial non-heme type enzymes perform halogenation without using any metal ions or

cofactors [68] whereas the eukaryotic non-heme type contain vanadium ion [3, 4, 86] and require it for halogenation [31]. Neither bacterial nor eukaryotic non-heme haloperoxidases are inhibited by azide [10, 38, 90].

Haloperoxidases function in the synthesis of key industrial chemicals. For instance, they can be utilized in the bromination of phenols to yield bromophenols and flame retardants, if the processes result in higher yields and provide a low cost approach [10, 61]. Current enzyme based applications include enzymatic chlorination of barbituric acid and halogenation of propylene. In the propylene halogenation mechanism the haloperoxidase reacts with propylene to generate a intermediate propylene halohydrin, which is oxidized to propylene oxide. In this process a halide ion is released and then reused. Using an enzyme based halogenation process is advantageous over the use of elemental halides (bromide, chloride, iodide, fluoride) because they are not pollutants or a safety hazard. Also, they are reusable, can give higher yields of products and are environmentally friendly [28].

Haloperoxidases are also used as agents in pollution prevention. The hypohalous acid intermediate formed by haloperoxidase in the presence of hydrogen peroxide and halide ion is used to kill microorganisms in process waters of pulp and paper mills [61].

Haloperoxidase enzymes including myeloperoxidase (MPO), lactoperoxidase (LPO) and eosinophilic peroxidase (EPO) are important in mammalian defense against microorganisms, parasites and tumors [61]. MPO and EPO produce hypohalous acid, halogens, and chloramines. LPO produces hypothiocyanous acid which kills oral bacteria and is used to prevent gingivitis, periodontal disease and tooth decay.

Thiocyanate and ureaperoxide are also used in powdered drugs to prevent their bacterial decomposition. All these substances exert their toxicity onto target cells, molecules of tumor cells, bacteria, parasite, fungi, and yeast infections.

Haloperoxidases have been isolated from marine algae and soil microorganisms [84]. Several naturally occurring bromoperoxidases have been isolated from soil bacteria such as *Streptomyces* and *Pseudomonas* and a number of marine algae [29, 46, 94, 96]. Although heme-type bromoperoxidases have been shown to be associated with the marine acorn worms *Notomastus lobatus* [16], *Ptychodera flavin laysancia*, *Thelepus setosus* and *Saccoglossus kowalevskii* [1], no non-heme bromoperoxidase has been isolated from acorn worms.

Acorn worms are soft-bodied benthic marine worms of the phylum Hemichordata and class Enteropneusta [96] which inhabit mucous lined burrows in shallow sediments [52]. Numerous halogenated metabolites have been isolated from their burrows. Several organohalogen compounds have been reported in acorn worm extracts [29]. Brominated secondary compounds (organobromines) present in the burrows are thought to function as signaling chemicals [29] but, their function as anti-microbial and anti-predation agents is under debate [29, 46, 82]. However, acorn worms have not been extensively studied for haloperoxidases production.

The focus of our study was to look for the presence of bromoperoxidases in bacteria associated with the Red banded acorn worms, collected from the Florida coast. Acorn worm body impressions and slime dilutions resulted in fifty different bacterial isolates. Isolate #4 was obtained from the slime dilutions. It is a Gram positive spore

forming rod. Biochemical tests place the bacterium in the genus *Bacillus* and the rDNA sequence shows 99% homology to *B. cereus*. Bromoperoxidase activity within this organism was initially detected by the qualitative Phenol Red Assay. The bromoperoxidase produced by isolate #4 was purified to 95% homogeneity using ion exchange chromatography and gel filtration. The protein was located in native PAGE gels by activity staining using the phenol red reagent. The monochlorodimedone assay (MCD) was used to quantitate activity after each step.

LITERATURE REVIEW

Halogenating enzymes are widely distributed in nature (marine and soil sources), occurring mainly as haloperoxidases. The structure, functional type, and the reaction mechanisms of haloperoxidases are of interest because they are linked to the biological significance of halogenated products such as antibiotics, anti-inflammables and anti-feedants (chemicals that inhibit feeding, for example, they prevent harmful insects from on crops).

Halogenation is the process of addition of a halogen (chlorine, bromine, fluorine or iodine) to a compound by an enzymatic reaction. One group of enzymes which catalyze this reaction are haloperoxidases. They halogenate substrates in the presence of halide ions and hydrogen peroxide [68]. Several haloperoxidases have been isolated from marine algae and soil microorganisms [10]. Numerous halogenated metabolites such as bromophenols and halogenated antibiotics have been isolated from the habitat (burrows) of hemichordates, commonly called the acorn worms, which are classified in several different genera. Thus, acorn worms are hypothesized to contain these halogenated metabolites [96]. Several organohalogen compounds have been isolated from whole body extracts of acorn worms [34]. However, acorn worms have not been extensively studied for haloperoxidase production. The halogenated metabolites are thought to be either (i) excreted by the worm itself, (ii) by its endogenous bacteria, (iii) by bacteria on the surface or associated with the worm and/or, (iv) by bacteria present in their burrows. No acorn worm slime (a layer of protective mucus coating on the body of

the worm) associated haloperoxidase-producing bacteria have been discovered and no haloperoxidase has been isolated and purified to date.

ACORN WORMS AND HALOPEROXIDASES

Acorn worms are marine worms of the phylum Hemichordata and class Enteropneusta [96]. They are commonly known as acorn worms. Acorn worms are soft-bodied benthic worms inhabiting mucus-lined burrows in shallow sandy marine sediments of estuaries, sub-tidal, and inter-tidal areas all across the country [52, 84]. *Saccoglossus kowalevskii* is a widely studied acorn worm [29] that produces organohalogens such as 2,4-dibromophenol (DBP) and several bromopyrroles [46]. The burrow walls of *S. kowalevskii* are lined with mucus containing DBP, which is found in high concentration at the open end of the burrow. DBP inhibits the microbial degradation of mucus which increases the longetivity of the mucus lining the burrow. The presence of DBP decreases aerobic activity in the burrow resulting in the formation of an ironoxyhydroxide layer due to the oxidation of ferrous iron [46]. This layer acts as a barrier to the influx of sulfide into the burrow and thus, stabilizes the burrow. Steward et al. [83] have isolated a debrominating Gram negative anerobic bacterium called DSL-1 from the extracts of *S. kowalewskyi* and *Balanoglossus aurantiacus*. DSL-1 debrominates 2,4,6-tribromophenol to 2,6-dibromophenol and 4-bromophenol. The nature of the association of this bacterium with acorn worms is not understood.

Organohalogens have been found in extracts of the acorn worms *Ptychodera flava laysanica* [33], *Ptychodera bahamensis* [18], *Notomastus lobatus* [84] and *Balanoglossus*

biminiensis and *aurantiacus* [6]. *P. flava layanica* produces 2,4,6 tribromophenol, 3-chloroindole, 3-bromoindole, and 6-bromo-3-chloroindole [33]. Higa et al. [33] suggest that 3-chloroindole and 3-bromoindole are breakdown products of tryptophan in a halide environment and are responsible for the iodoform-like odor given by the mucus secretions of these acorn worms. The volatile extracts produced by *P. bahamensis* contain twenty halogens: twelve brominated aromatics, five aromatic compounds containing both chlorine and bromine, two chlorine containing nitrogenous compounds, and one chlorophenol, which is the first to be reported from an acorn worm [18]. *N. lobatus* produces the volatile halogens 4-bromophenol, 2,4-dibromophenol, and 2,4,6-tribromophenol. 2,6 and 2,4-dibromophenol cause the iodoform-like odor associated with *B. biminiensis* and *B. aurantiacus* [6].

Brominated compounds (organobromines) present in the burrows of acorn worms and in high concentration in the tail of the worms are thought to be signaling chemicals for a defensive function. Woodin et al. [96] suggest that the brominated compounds protect the worm against attack by microorganisms and large predators. Since these compounds are most concentrated near the burrow opening [84] and are volatile and toxic, they are thought to function as deterrents to marine predators [29, 96]. The precise function of these organobromines as anti-microbial and anti-predation agents, however, is under debate [29, 46, 82].

The mechanism by which these worms produce halogenated compounds, especially organobromines, has not been investigated and is not well understood. Two hypotheses regarding the presence of these halogenated compounds state that either the

acorn worms obtain these brominated compounds from their diet or that these compounds maybe from anthropogenic pollutants such as pesticides [29]. Also, the isolation of a bacterium from acorn worm extracts by Steward et al. suggests that bacteria in their habitat or associated with them may be responsible for halogenated products [83].

HALOPEROXIDASES

Haloperoxidases are enzymes that catalyze the oxidation of halide ions in the presence of hydrogen peroxide [10, 56, 60]. The halide ion (X^-) in the reaction below is chloride, bromide, or iodide. Hydrogen peroxide reacts with the electrophilic halide which then reacts with organic substrates [60].

$$X^- + H^+ + H_2O_2 + R_2CH \longrightarrow R_2CX + 2H_2O \quad [3]$$

Haloperoxidases are divided into three groups based upon their halide specificity: iodoperoxidases, chloroperoxidases and bromoperoxidases [10]. They are named according to the most electronegative halide that can be oxidized in the presence of hydrogen peroxide [10]. Thus, in the presence of hydrogen peroxide, chloroperoxidases are enzymes that oxidize chloride, bromide or iodide. Oxidation of bromide and iodide is catalyzed by bromoperoxidases and iodoperoxidases catalyze only the oxidation of iodide [10]. The $I^- > Br^- > Cl^-$ preference, known as the Hofmeister lyotropic series of affinity, corresponds to the decreasing alkalinity of residues near the active center of the enzyme [21].

There are three types of haloperoxidase enzymes: the heme type, the bacterial non-heme type, and the eukaryotic non-heme type [10]. The heme type haloperoxidases

have a protoporphyrin IX as their prosthetic group [32, 90] and are inhibited by azide [10]. They can also be identified by the presence of a soret band (the absorption band of all porphyrins) at 399-405 wavelength [48]. In addition some heme haloperoxidases also show faint staining with benzedene [48]. The bacterial non-heme type enzymes perform halogenation without the requirement of metal ions or cofactors [68] whereas, the eukaryotic non-heme type contain vanadium ion [3, 4, 86] and require it for halogenation [31]. Vanadium bromoperoxidase (V-BrPO) contain a vanadate ion as a prosthetic group and catalyze the formation of a carbon bromine bond in the presence of hydrogen peroxide, bromide ion, and bromide acceptor [75]. Neither bacterial nor eukaryotic non-heme haloperoxidases are inhibited by azide [10, 38, 90]. The bacterial non-heme bromoperoxidases have recently been classified as perhydrolases by vanPee et al. [90]. These enzymes use carboxylic acids for halogenating activity to form peracids. Specifically, they act as perhydrolases in the presence of hydrogen peroxide by hydrolyzing the acyl enzyme intermediate to form peracid which oxidizes halide ions to form hypohalous acid. The acid then halogenates or oxidizes the organic substrates.

Eukaryotic non-heme type haloperoxidase (vanadium haloperoxidase)

Eukaryotic non-heme type marine haloperoxidase known at present include bromoperoxidase from several algae and a few fungi (Table 1).

Table 1: Eukaryotic non-heme marine haloperoxidases.

ORGANISM NAME	KINGDOM	REFERENCE(S)
I. Bromoperoxidase		
Penicillus capitatus	Green alga	62
Halimeda sp.	Green alga	10
Carollina officinalis	Red alga	77
Carollina pilulifera	Red alga	36, 78
Carollina vancouveriensis	Red alga	76
Ochtodes secundiramea	Red alga	73
Ecklonia stolonifera	Red alga	31
Ceramium rubrum	Red alga	51
Alaria esculenta	Brown alga	49
Ascophyllum nodosum	Brown alga	15, 20
Chorda filum	Brown alga	19
Laminaria sacchirina	Brown alga	3, 19
Laminaria digitata	Brown alga	43
Laminaria hyperborea	Brown alga	4
Laminaria ochroleuca	Brown alga	4
Pelvetia canaliculata	Brown alga	3
Xanthoria parientina	Lichen	21
II. Iodoperoxidase		
Saccorhiza polyschides	Brown alga	2
Phyllariopsis brevipes	Brown alga	2
Laminaria saccharina	Brown alga	2
Laminaria hyperborean	Brown algae	2
Pelvetia canaliculata	Brown alga	2
III. Chloroperoxidase		
Curvularia inequalis	Fungus	72

The halogenating enzymes from the brown alga *A. nodosum* and the red alga *C. offinalis* and *C. inequalis* have been the well characterized and their crystal structures have been determined [19,20]. Vanadium bromoperoxidase (V-BrPO) of *C. pilulifera* has been cloned and expressed in *E. coli* [13] and *Saccharomyces cerevisiae* [65].

Halogenation reactions catalyzed by V-BrPO in the presence of hydrogen peroxide and bromide ion include: conversion of alpha amino acids and peptides to

nitriles and aldehydes by the V-BrPO of *P. capitatus* [62], formation of bromomethanes such as bromoform by the BPO isolated from *C. pilulifera* [36, 40, 51, 63], biosynthesis of monoterpenes by V-BrPO of *O. secundiramea* [87], bromination and cyclization of terpenes, and terpene analogus by V-BrPO of *Rhodophyta* [14].

The bromoperoxidase activity of the VBrPO isolated from the marine green macro-alga *Ulvella lens* is unique, because its activity is enhanced but does not require the addition of cobalt or vanadium ions [64]. This V-BrPO also catalyzed the halogenation of oxaloacetate to form dibromomethane and tribromomethane without the addition of cobalt or vanadium.

X-ray structure

The X-ray structure of the vanadium chloroperoxidase (V-ClPO) from *Corallina inequalis* has been determined by Butler et al. [11]. The main structural motif of the native form of V-ClPO at 2.03Å resolution consists of four helix bundles. Vanadate is ligated to the haloperoxidase by a histidine in a pentagonal bipyramidal geometry. Vanadate is located at the top of one of the helix bundles in a channel lined by polar residues along with main chain carbonyl oxygen atoms on one side and hydrophobic residues on the other side. In this channel, vanadate is ligated to the haloperoxidase by three oxygen atoms in the equatorial plane. Here vanadate is hydrogen bonded to the positively charged residues, lysine, arginine and serine and an amine nitrogen proton of glycine. An apical hydroxide is hydrogen bonded to an acid –base histidine. The X-ray structure of the peroxide form of vanadium chloroperoxidase (V-ClPO) at 2.24Å resolution is a distorted tetragonal pyramid. In this form the peroxidase coordinates

vanadium in a side-on bound fashion with an oxygen (V=O) instead of hydroxide and is not hydrogen bounded to histidine. Instead one peroxide oxygen is hydrogen bonded to lysine. Histidine and an oxygen atom are in the basal plane of the vanadium and an oxoligand is in its axial plane.

This V-ClPO has an active site similar to the V-BrPO of *A. nodosum*. The X-ray structure of *A. nodosum* has been determined at 2.0Å resolution [53, 95]. It consists of four helix bundles and three small beta sheets with a trigonal bipyramidal vanadium site. V-ClPO and V-BrPO, both have the same vanadate binding sequence motif. However, V-BrPO has an extra histidine in its active site.

Crystal structure of the V-BrPO of *C. officinalis* at 2.3Å folds into a single alpha and beta domain [74, 35]. There are twelve BPO subunits each with a cavity in the center of the dodecamer formed by the N-terminus of the subunit. The subunit fold is similar to that of *A. nodosum* enzyme and residues in the active site involved in vanadate binding are conserved between the V-BrPO of *C. officinalis* and *A. nodosum*, and the V-ClPO of *C. inequalis*. The overall sequence identity between these enzymes is only 33% but the location and coordination of vanadate in the active center of all vanadium haloperoxidases is the same. Vanadate is hydrogen bonded to a histidine in the active center and surrounded by positively charged amino acids. The overall structure between haloperoxidases differs in the number of alpha and beta subunits.

Halogenation reaction mechanism

The bromination or chlorination reaction mechanism of vanadium haloperoxidase (V-HPO) is similar to the reaction mechanism of the heme and the bacterial non-heme type haloperoxidase. The peroxide from hydrogen peroxide binds vanadium of the V-HPO to form a peroxovanadium species or compound [5, 91]. The two dominating peroxovandate are mono $(HVO_2(O_2)_2^{2-})$ and di $(HVO_2(O_2)_2^-)$ protonated bis (peroxo) vanadates in protonation or deprotonation equilibria with each other [5, 67]. The protonated peroxovandate undergoes a nucleophilic attack by bromide and bromide is oxidized. The oxidized bromide binds to a carbon in the vicinity of the vanadium. This results in the formation of an intermediate, mainly hypohalous acid, which halogenates an organic substrate to form halocompounds or halohydrins. The hypohalous acid or the enzyme-bromine intermediate can also oxidize a second equivalent of hydrogen peroxide to from dioxygen [22, 91].

Regioselectivity

The V-BrPO of *A. nodosum* and *C. offinalis* catalyze regioselective halogen-dependent oxidation of indoles [11, 58]. 1,3-di-tert-butylindole is converted to 1,3-di-tert-butyl-2-inolinone by V-BrPO in the presence of hydrogen peroxide and bromine, at pH 6.0. The first bromoindoliniom species is formed by the electrophilic attack of the brominating species on the electron rich carbon (C2) to carbon (C3) double bond of the pyrrole ring. This is followed by hydration at the C2 position. The relatively hydrophobic channel of the VBrPO with only three hydrophilic residues leads to substrate selectivity and specificity for the direct oxidation of indoles.

Vanadium haloperoxidase also perform enantioselective sulfoxidation [5, 79, 86]. The mechanism of conversion of bicyclic sulfide to asymmetric sulfoxide is similar to halide oxidation with one exception. The acid equivalent required for reactivity in halide oxidation is consumed in each cycle and has to be replenished, whereas, in sulfoxidation the acid is not consumed. Some examples of sulfoxidation include, formation of (R)-methyl phenyl sulfoxide with 91% enantiomeric excess by the V-BrPO of *A. nodosum*, (S)- methyl phenyl sulfoxide formation in 58% enantiomeric excess by *C. pilulifera*, and sulfoxidation of indenes to the *S-sulfoxide* form by *C. officinalis* V-BrPO [17, 86].

Bacterial non-heme type haloperoxidases

Most of the known bacterial non-heme type bromoperoxidases (BPO) have been isolated from non-pathogenic soil microorganisms. One of the earliest known bacterial non-heme bromoperoxidase was isolated in 1984, from the chloramphenicol producer *Streptomyces phaeochromogenes* [88]. Since then, bromoperoxidases have been isolated from several *Streptomyces* species, many of which also produce halogenated antibiotics. However, no correlation between BPO and antibiotic production by any microorganism has been established. Table 2 lists several non-heme bromoperoxidase.

Table 2: Bacterial non-heme haloperoxidases. Molecular weight is given in killodaltons (kDa).

MICRORGANISM		MOLECULAR WEIGHT (kDa)	REFERENCE(S)
I. Bromoperoxidase (BPO)			
Streptomyces griseus	BPO 1a* BPO 1b* BPO 3*	70 90 90	97
Streptomyces aureofaciensTü24	BPO-T*	-	89
Streptomyces venezuelae ISP5230	BPO- catalase	-	24
Streptomyces aureofaciens ATCC 10762	BPO-A1* BPO-A2*	65 90	50, 94
Pseudomonas pyrrocinia	BPO with no prosthetic groups	-	37
Pseudomonas pyrrolnitrica	4 BPO isoenzymes	-	47
Pseudomonas putida IF-3 strain	Cobalt (Co) activated BPO- EST(esterase) BPO	68 -	37, 39, 41
Pseudomonas aeruginosa	BPO	-	37
Serratia marcescens	BPO	-	71
Shigella flexneri	BPO	-	85
Salmonella enterica ser. typhimurium	BPO	-	85
Acinetobacter calcoaceticus F46	BPO	-	44
Rhodococcus erythropolis NI86/21	BPO	-	23
II. Chlopoperoxidase (CPO)			
Pseudomonas pyrrocinia	CPO-P	-	98
Pseudomonas flourescens	CPO-F	-	47
Streptomyces lividans TK64	CPO-L	-	8
Serratia marcescens W250	CPO	29	71

* **Anotation provided by the author for different bromoperoxidases.**

- means that the molecular weight has not been determined.

To distinguish the BPO obtained from different *Streptomyces* species, the enzymes are annotated by the authors, such as BPO-T, BPO-A1, BPO-A2. The N-

terminal amino acids of BPO-T (Table 2) have been sequenced [89] and the bpoT gene was used as a probe for BPO-A1 and BPO-A2 identification. The genes for BPO-A1 and the BPO-A2 have been cloned into *S. lividans* TK64 [67, 69]. The bpoA2 gene has 90% homology with the bpoT gene. For chloroperoxidase, the letters P, L and F are used to distinguish chloroperoxidases isolated from different microorganisms (Table 2). CPO-L exhibits 71% homology with CPO-P of *P. pyrrocinia* [93] and 42% homology with BPO-A2 of *S. aureofaciens* [94]. CPO-F enzyme is thought to lack substrate and regiospecificity. The non-heme CPO produced by *S. marcescens* W250 is the first haloperoxidase to exhibit phosphatase activity [71].

All the bacteria included in Table 2 produce haloperoxidase enzymes and halogenated metabolites except for *S. lividans* TK24, which has chloroperoxidase (CPO-L) activity but has not been reported to produce halogenated metabolites [8]. This maybe due to the small amount of CPO-L produced by *S. lividans* such that enzyme purification can only be accomplished after overexpressing the enzyme in *S. aureofaciens* Tü24-88. The bromoperoxidase from *S. aureofaciens* ATCC 10762 has been crystallized using the hanging drop method [80]. The crystal structure was resolved at 2.3Å and the protein has a molecular weight of 60,200 Da. The active center of the enzyme is characterized by a serine hydrolase motif.

Pseudomonas species are also responsible for the production of many different bromoperoxidases (Table 2). *P. pyrrocinia* contains a non heme BPO with no metal prosthetic groups [37]. The organism synthesizes the chlorine containing halogenated antibiotic pyrrolnitrin [47] but the relation, if any, between the BPO and the antibiotic

production is not known. The non-heme BPO isolated from the IF-3 strain of *P. putida* contains the metal ions cobalt, zinc, nickel and ferric iron and is a novel non-heme metal containing BPO [39, 41]. The metal ion content of this 68 kDa enzyme was determined by inductively coupled plasma atomic spectrometry–mass spectrometry (ICP-AES-MS) and high performance liquid chromatography (HPLC) [39]. Itoh et al. [39] used polyacrylamide gel electrophoresis (PAGE) and iso-electricfocusing (IEF) to identify the two 33 kDa subunits. This BPO-EST enzyme refutes the previously described classification of the types of haloperoxidases, as it is a novel bacterial non-heme BPO dependent on a cofactor cobalt and organic acids like n-butyric acid, propionic acid, or isobutyric acid in addition to hydrogen peroxide and bromide, to exhibit its halogenation properties [37].

Itoh et al. [41] cloned and characterized this cobalt activated BPO-esterase (BPO-EST) gene of *P. putida* IF-3 strain. The enzyme belongs to the serine hydrolase family and contains the "Gly-X-Ser-X-Gly" motif [41] in its active site. The esterase of BPO-EST shows substrate specificity towards (R)-acetylthioisobutyric acid methyl ester [41]. Bromination by the combined BPO-EST unit results in racemic mixtures of bromohydrins. The *P. putida* IF-3 strain also has a single non-heme BPO which catalyzes the substrate specific oxidation of aniline by a mechanism similar to the previously described mechanism by Itoh et al. [38]. The non-heme BPO shows substrate specificity for aniline oxidation in the presence of hydrogen peroxide and bromide ions. Aniline's reaction with hydrogen peroxidase in the presence of bromide ions results in the formation of a peroxo intermediate. This intermediate oxidizes aniline to give o- and

p-bromoaniline, azobenzene, azoxybenzene, and nitrobenzene as products. In the absence of bromide ions the oxidation of aniline does not form o-and p-bromoaniline.

The reaction mechanism of this cobalt activated BPO-EST from *P. putida* IF-3 has been further studied by Kawanami et al. [45]. The bromination reaction occurs with the participation of the serine residue from the catalytic triad and the formation of free peracetic acid intermediate (depending upon the hydrogen peroxide concentration) as shown for other non metal haloperoxidases. In this reaction mechanism, nucleophilic attack by the hydroxyl group of the serine residue (from the catalytic triad motif) produces an acyl intermediate between the enzyme and acetic acid. The carbonyl of the acyl intermediate is attacked by a peroxoanion formed by the coordination of hydrogen peroxide by the cobalt ions. This results in an enzyme-peroxy intermediate which is stabilized by cobalt. In the presence of bromine this intermediate is oxidized to give free hypobromous acid and acetic acid is released. In the absence of cobalt, in a perhydrolysis reaction between the enzyme and acetic acid excess hydrogen peroxide liberates a small amount of free peracetic acid. Bromination by this chemical reaction is several times slower than bromination by the cobalt activated enzymatic reaction. In the absence of bromine, aniline, and methyl p-tolyl sulfide are oxidized by the enzyme-peroxy intermediate. The contribution of free peracetic acid to organic compound oxidation is negligible. Therefore, the enzyme-peroxy intermediate is the intermediate used in halogenation and free peracetic acid is only involved in a side reaction.

Pelletier et al. [68] studied the halogenation mechanism of bacterial non- heme haloperoxidases and compared it with that of esterase of *P. flourescens* and other serine

hydrolases and concluded that a catalytic triad of serine, histidine, and aspartate is required for halogenation. The catalytic triad defined as the "Gly-X-Ser-X-Gly" motif [68] is present in bacterial non-heme BPO and esterase. This places the enzyme in the serine hydrolase family containing the ά/β hydrolase fold. The serine hydrolase mechanism for the esterase involves nucleophilic attack by the hydroxyl group of the serine in the active site on the carbonyl group of the substrate, resulting in ester-hydrolysis. Similarily, in the halogenation reaction serine in the active site reacts with acetate to from a serine/acetate ester. The ester is hydrolyzed by hydrogen peroxide to form the intermediate peracetic acid. Peracetic acid being a strong oxidizing agent oxidizes the bromide ion to from hypohalide ion. Hypohalide ions act as halogenating agents or oxidizers of aromatic amino groups to nitro groups. Pelletier et al. [68] concluded that the serine- hydrolase motif is found in the conserved region of all bacterial non-heme haloperoxidase.

Heme-type haloperoxidase

The heme type marine haloperoxidases discovered so far are listed in Table 3. Most heme-type haloperoxidase show halogenation, peroxidase and catalase activity and their lack of substrate specificity [10] is currently debated.

Table 3. Heme-type marine haloperoxidases

ORGANISM NAME	TYPE OF ENZYME	REFERENCE (S)
I. Green algae		
Penicillus capitatus	Bromoperoxidase (BPO)	7
Penicillus lamourouxii	BPO	7
Rhipocephalus phoenix	BPO	7
II. Red algae		
Cystoclonium purpureum	BPO	26
Rhodomela larix	BPO	1
III. Fresh water alga		
Cladophora glomerata	BPO	92
IV. Marine worms		
Notomastus lobatus	BPO	16
Ptychodera flavin laysanica	BPO	1
Thelepus setosus	BPO	1
Saccoglossus kowalevskii	BPO	1
V. Fungus		
Caladaromyces fumago	CPO	27
VI. Bacteria		
Streptomyces griseus Tü6	BPO2	92
Streptomyces venezuelae ISP5230	BPO-catalase	57
Streptomyces toyocaensis NRRL15009	BPO-catalase CPO	57
Pseudomonas pyrrolnitrica	BPO-catalase	83
Pseudomonas putida	BPO-catalase	83
Pseudomonas aeruginosa	BPO-catalase	83
Pseudomonad EF group 70B strain	BPO-catalase	54
VII. Others		
Mammalian white blood cells	Myeloperoxidase Eosinophil peroxidase	66 10

The haloperoxidase obtained from *Cladophora glomerata* [92] contains a 43 kDa enzyme that catalyzes the iodination of tyrosine, oxidation of iodine and guaiacol in the presence or absence of chloride or bromide ions [Table 3]. The BPO-catalase from *S. venezuelae* [57] exhibits bromoperoxidase, catalase and a small amount of peroxidase

activity. In contrast to other heme BPO's, this enzyme resembles catalases more than bromoperoxidases. *P. pyrrolnitrica* has a heme-type catalase-bromoperoxidase with both catalase and peroxidase activity. Heme type catalase-BPO of molecular weight 68 kDa and 86 kDa have also been isolated from *P. putida* and *P. aeruginosa*, respectively [37]. Kuusk et al. [54] isolated a heme BPO-catalase from a *Pseudomonad* EF group 70B bacterial strain isolated from the recycled pulp white water. This enzyme shows high catalase activity and a native molecular weight of 153 kDa. However, the N-terminal amino acid sequence does not have any homology with other known catalase or peroxidase sequences. Enzymes which have catalase and peroxidase activity are sometimes referred to as KatGs.

The haloperoxidase activity of these catalase-peroxidase (KatGs) has been described for the first time by Jakopitsch et al. [42]. The KatGs enzyme isolated from the cyanobacterium *Synechocystis* has been shown to exhibit halogenated chlorination and bromination and has been classified as a heme haloperoxidase.

Among the heme haloperoxidases, the crystal structure of the CPO from *C. fumago* has been determined [27] along with the X-ray structure of the canine myeloperoxidase at 3Å resolution [25, 27]. Both their tertiary structures contain eight helical segments. The heme is sandwiched between the two domains of this highly alpha helical protein. In the catalytic triad the histidine is replaced by glutamic acid.

The reaction mechanism for halogenation is the same as for the non heme or the vanadium haloperoxidases. However, the intermediate free hypohalous acid previously detected and thought to be the mechanism for halogenation is being debated. Recently,

the intermediate detected by UV spectroscopy is the hypohalous adduct of iron(III) protoporphyrin(IX) [55]. This intermediate is hypothesized to react with specific substrates.

HALOGENASE

Halogenases are $FADH_2$-dependent halogenating enzymes with a "Gly-X-Gly-X-X-Gly" nucleotide binding motif [90]. Halogenases utilize $FADH_2$ and oxygen to perform a monooxygenase-type reaction during which oxygen activates the organic substrate. This substrate then catalyzes a regioselective nucleophilic attack by an enzyme bound halide where the halide ion incorporation position depends upon the regioselectivity of the halogenase [90].

Halogenase genes have been identified in the biosynthetic gene clusters for halogenated metabolites such as pyrrolnitrin, 7-chlorotetracycline, pyoluteorin, pentachloropseudilin, rebeccamycin, thienodolin, and the vancomycin group antibiotics chloroeremomycin and balhimycin [90]. All contain two conserved tryptophan residues, which might be involved in halide binding [91].

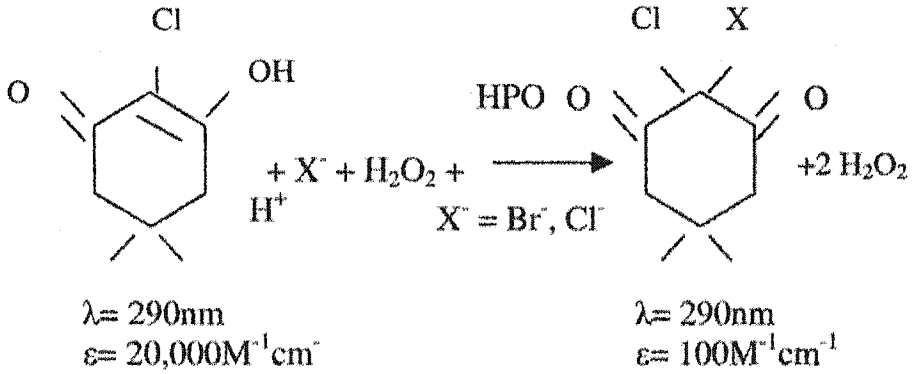
The genes for $FADH_2$-dependent halogenase can be detected by genetic probes. Degenerate primers for the bacterial halogenase gene fragment of *Streptomyces venezuelae* ISP5230 have been generated by Piraee et al. [70]. The PCR fragment generated by these primers showed sequence homology with halogenase genes from *S. aureofaciens* NRRL2209 and *S. coelicolor* A3 [2]. The PCR method is faster than Tn5 mutagenesis or shotgun cloning previously used to clone halogenase genes. However, the

primer design for the PCR method needs to account for specific codon usage for added specificity and efficiency. This is difficult because of the variability in the halogenase gene from each microorganism. Therefore, a PCR product implies the presence of a putative halogenase gene and no PCR product does not eliminate the possibility of the presence of the halogenase gene. Also, the halogenating activity of halogenases can only be shown if its natural substrate or a substrate with similar structure is known [91] as there is no chemical reaction to correlate halogenase gene with halogenase activity.

ASSAYS FOR HALOPEROXIDASE ACTIVITY DETECTION

Monochlorodimedone (MCD) Assay

Monochlorodimedone (MCD) is a spectrophotometric assay used to detect haloperoxidase activity [59]. MCD know as 2 chloro-5,5-dimethyl-1,3-dimedone, is a classic organic substrate used to measure haloperoxidase activity. The reaction mechanism is shown below [12]:



The standard assay was developed by Hager et al. [30], where they used 42 M MCD, 8.8mM hydrogen peroxide, 10mM sodium azide in 1M sodium acetate buffer at pH 5.5 and room temperature. The enzyme amount is adjusted so that the average length of the assay is 30 minutes. The consumption of MCD by the haloperoxidase is detected by the decrease in absorbance at 290nm over time. The conversion of one micromole of MCD in one minute by the enzyme is defined as one Unit (U) of enzyme activity [10]. MCD assay is therefore, a quantitative assay for the measurement of haloperoxidase total and specific activity.

Phenol Red Assay

The Phenol Red assay is a colorimetric assay used for haloperoxidase screening [28]. In the Phenol Red assay, phenol red (phenolsulfonepthalein) is converted to bromophenol blue (tetrabromophenolsulfonepthalein) in the presence of hydrogen peroxide [87]. The hydrogen peroxide acts on the metal (in this case, bromine) to form a peroxometal species. This oxidized bromine species brominates phenol red. Other halides oxidized by hydrogen peroxide are chlorine and iodine which can then chlorinate or iodinate phenol red. Haloperoxidases, such as BPO, use phenol red as their substrate and a positive reaction is characterized by yellow to blue color change [87]. Phenol red absorbs at 429 nm and bromophenol at 598nm. Phenol red conversion can also be measured at an optical density of 598 nm. Phenol red assay is a qualitative assay used for screening and can be used to detect enzyme activity in unpurified protein samples.

SIGNIFICANCE OF HALOGENATION AND HALOPEROXIDASES

The biological importance of halogenated products ranges from defense mechanism chemicals for organisms and catalysts in halogenation reactions to pharmacological drug synthesis and anti-inflammatory activities [61]. Naturally occurring halometabolites include antibiotics such as chlorotetracycline, chloramphenicol, griseofulvin, and pyrrolnitrin; other antimicrobial agents such as phenols, avrainvilleol, methyl alkyl, and alkyl vinyl ketones; anti-inflammables such as griseofulvin; antifeedants such as avrainvilleol and many others [61]. The halogenated organic compounds are more toxic than their organic counterparts. For example, chloramphenicol is more potent compared to its non potent chlorinated derivate corynecin.

Haloperoxidase enzymes including myeloperoxidase (MPO), lactoperoxidase (LPO)and eosinophilic peroxidase (EPO) are important in mammalian defense against microorganisms, parasites and tumors [61]. MPO and EPO produce hypohalous acid, halogens and chloramines. LPO produces hypothiocyanous acid which kills oral bacteria and is used to prevent gingivitis, periodontal disease and tooth decay. Thiocyanate and ureaperoxide are also used in powdered drugs to prevent their bacterial decomposition. All these substances exert their toxicity onto target cells and/or molecules of tumor cells, bacteria, parasite, fungi and yeast infections.

Haloperoxidases also function in the synthesis of key industrial chemicals. For instance, they can be utilized in the bromination of phenols to yield bromophenols and flame retardants in processes which result in higher yields and provide a low cost

approach [10, 61]. Current enzyme-based applications include enzymatic chlorination of barbituric acid and halogenation of propylene. In the propylene halogenation mechanism, haloperoxidase reacts with propylene to generate a intermediate propylene halohydrin. In this process a halide is released and then reused. Using an enzyme based halogenation process is advantageous over the use of elemental halides (bromide, chloride, iodide, fluoride) because they are not pollutants or a safety hazard. Also, they are reusable, can give higher yields of products and are environmentally friendly [28].

Haloperoxidases are also used as agents in pollution prevention. The hypohalous acid intermediate formed by haloperoxidase in the presence of hydrogen peroxide and halide ion is used to kill microorganisms in process waters of pulp and paper mills [61].

SUMMARY

More than 700 halogenated organic compounds have been found in natural sources, mainly marine environments. These halogenated compounds are synthesized by haloperoxidases which act as biocatalysts or enzymes. The haloperoxidases catalyze similar reactions but vary in their molecular weights, amino acid number, and sequence and substrate specificity. Each of these enzymes also has specific and unique reaction requirenments. A large number of haloperoxidases have been isolated from microorganisms, however, others have been reported from algae, fungi and hemichordates. Most of these organisms produce more than one haloperoxidase.

The mechanism of the addition of a halide ion into an organic compound remains controversial. However, the formation of hypohalous acid as the intermediate is essential

in all reactions. Here, the concentration of the substrates sometimes dictate the product formed. For example, increasing the concentration of hydrogen peroxide, in a reaction with methyl acetylene, shifts the production of monohalogenated products to the synthesis of dihalogenated products. In another example, allyl alcohols form bromohydrins in low bromide ion concentration but produce dibromoderivatives in high bromine concentrations. Structurally, all haloperoxidases have a catalytic triad including serine in the active site and have been placed in the alpha beta hydrolase fold enzyme family.

Substrate specificity and regioselectivity of haloperoxidases is also under debate. According to van Pee, these enzymes lack selectivity and specificity and thus are least likely to be involved in the biosynthesis of halogenated metabolites. However, haloperoxidases have been isolated from numerous marine species responsible for the production of halometabolites. Also, the regioselectivity of vanadium bromoperoxidases has been established for a number of substrates for halogenation and sulfoxidation reactions as described previously. Substrate specificity has also been established for *P. putida* IF3 strain's non-heme BPO.

The halogenase gene seems to be required for the synthesis of many of the known antibiotics [91]. For example, pyrrolnitrin produced by *P. flourescens*, a glycopeptide produced by *Amycolatopsis mediterranei*, bromobalhimycin, and chlorobromobalhimycins made by *A. balhimycina,* and chloramphenicol isolated from *S. venezuelae*. More research is needed to determine if that the halogenase gene is responsible for halogenation.

In conclusion, many halometabolites with industrial or biomedical uses have been isolated from natural sources, especially from the marine environment. Haloperoxidases are one of the major group of enzymes involved in the synthesis of these compounds. The reaction mechanisms of these enzymes show similarity while the halogenated products generated vary by the substrate used and the environment from which the haloperoxidase originates. Also, the reaction conditions, enzyme concentration and the amount of hydrogen peroxide influence the enantioselectivity of the product produced. All this leads to many variables in the production of halogenated products by haloperoxidases and more study is needed to specify specific reaction conditions to obtain the desired products.

The controversy on the substrate specificity and selectivity of haloperoxidases (according to vanPee's research, they are not substrate specific and selective) is currently debated by many researchers in this area. The function of the halogenase gene (present in many antibiotic-producing organisms) and its relationship with the halogenation process has to be further explored to understand the substrate specific halogenation by the halogenase enzyme. At present, this research is restricted by the requirement of the knowledge of the substrate used for halogenation. The PCR approach to finding the halogenase gene is restricted by the increasing variables in halogenase gene sequence from organism to organism.

In the quest for enhanced understanding and knowledge of the halogenating process and of the organisms that produce these enzymes, we have isolated a marine bacterium associated with the slime of an acorn worm. This bacterium produces a BPO

which we have isolated and studied. To our knowledge, no other studies have focused on bromoperoxidase-producing bacteria in the marine environment and in association with the slime layer of the acorn worm. We intend to purify the enzyme from our isolate and characterize the bacterium and its bromoperoxidase further.

Studying the marine environment of the acorn worm, the microorganism associated with halogenation and the BPO enzyme will enrich our understanding about the types, specificity and potential industrial use of these enzymes. Substituting haloperoxidases for elemental bromine, chlorine, or iodine in the synthesis of organohalogens, is a step towards "green chemistry," that is, environmentally friendly chemical technology.

MATERIALS AND METHODS

Isolation of the bromoperoxidase producing bacteria from the acorn worm

The acorn worm body was scraped using a sterile glass rod to remove the slime layer. The slime was then dissolved in 0.1 M sterile saline and serial ten fold dilutions of the slime were plated onto Marine Agar 2216 [97]. Impressions of the worm body were made onto Marine Agar 2216 by rolling the worm onto autoclaved 5 by 7 inch rectangular foil containers containing agar. These foil containers were covered with autoclaved foil and incubated at room temperature for 3 days. The colonies obtained from both methods were streaked for isolation. Stock agar slants of the isolates were stored under mineral oil at 4°C. The agar slant morphology was used to group bacteria on the basis of slant color, texture and slant edges. Many of these bacterial isolates had a characteristic iodine-like odor including isolate #4. However, isolate #4 was arbitrarily chosen for further study.

Bacterial characterization

Biochemical tests

Biochemical tests were performed on isolate #4 by using api20NE and the api20E strips by bioMérieux (Apbiotech, Marcy l'Etoile, France). Both strips in general are not used to identify gram positive rods. However, they were used because multiple tests could be performed simultaneously.

16S rDNA amplification, sequencing, and phylogenetic analysis

The genomic DNA of isolate #4 was extracted using the Qiagen (Valencia, CA) genomic tip 20/G kit. One milliliter(ml) of isolate #4 bacterial culture was pelleted, lysed, bound to a column, washed, and the DNA was eluted with elution buffer. Isopropanol precipitation of the DNA resulted in high molecular weight genomic DNA. For polymerase chain reaction (PCR), this high molecular weight DNA was sheared using a sterile syringe.

PCR on the bacterial DNA was performed using 16S ribosomal DNA universal bacterial primers [32]. The reaction conditions were an initial denaturation step at 92°C for 2 minutes, 30 cycles of amplification with each cycle consisting of denaturation at 95°C for 15 seconds, annealing for 30 seconds at 55°C, and DNA extension for 2 minutes at 72°C. The 30 cycles were followed by a final extension for 6 minutes at 72°C. The PCR product was visualized on a 1% agarose gel and stained with ethidium bromide. It was purified with the Qiagen PCR purification kit to remove excess primers and dNTPs . The purified PCR product was sent for sequencing to Sequetech Corporation (Mountain view, CA). The universal primers used for DNA amplification were reapplied for sequencing. A BLAST search on the sequenced DNA was performed using VectorNTI and a phylogenetic tree was constructed with the aid of VectorNTI and ClustalW software.

Bacterial growth

A bacterial growth curve was obtained by growing isolate #4 in 1 liter (L) of Marine Broth 2216 (agar excluded) on a room temperature shaker for 72 hours. Samples (1 ml) were collected every hour and dilutions were plated onto Marine Agar 2216. The number of colony forming units (CFUs/ml) were calculated and used to generate a growth curve. A 1ml bacterial culture sample was removed every hour. BPO protein was partially purified from each 1ml sample and assayed for bromoperoxidase production. The protein was partially purified by cell sonication, supernatant collection, anion exchange column chromatography and concentration, as described in the section on protein purification. Phenol red assay was performed to compare growth with bromoperoxidase activity. The bacterial growth was stopped when sporulation was observed in a wet mount of the bacterium.

Streptomyces aureofaciens ATCC 10762 was the positive control for bromoperoxidase activity. It was grown on a mineral salt medium [96]. The *S. aureofaciens* bromoperoxidase protein was partially purified by the same method described for isolate #4.

Protein purification

Sonication

Two liters of isolate #4 bacterial culture grown in Marine Broth 2216, was pelleted by centrifugation using a Sorval RC2-B centrifuge and the GSA rotor at 4°C, 6000 g for 15 minutes. The cell pellet was resuspended in Tris-SO_4 buffer (0.03M Tris-H_2SO_4, 0.3M NaCl, pH 8.3) and cells were pelleted again by centrifugation at 4°C, 10,000g for 15 minutes. The pellet was resuspended in 10 ml Tris-SO_4 buffer (0.03M Tris-H_2SO_4, 0.3M NaCl, pH 8.3).

A Branson sonic power Sonifier cell disruptor 350 (Branson Ultrasonics corp. Danbury, CT) was used for sonication. Eight 30 second pulses at output control 4, 40 W, and 50% duty cycle were performed. The suspension was kept on ice during the procedure. Samples of the sonicated suspension were examined under a phase contrast microscope to determine the extent of cell disruption. Cell debris in a wet mount indicated complete cell disruption. The suspension was then centrifuged (Sorval RC2-B, SS-34 rotor) at 4°C, 10,000g for 20 minutes. The supernatant was clarified by passing it through a 0.2μm Acrodisc PF syringe filter (Millipore, Billerica, MA).

Anionic exchange chromatography

A Hi Trap Q HP column was used for anionic exchange chromatography (Amersham Biosciences, Piscataway, NJ). Before application of the lysate, the column was equilibrated with a step-wise application of 5ml 0.3M NaCl buffer (0.03M Tris-H_2SO_4, 0.3M NaCl, pH 8.3), 1.0M NaCl (0.03M Tris-H_2SO_4, 1.0M NaCl, pH 8.3), and

0.3M NaCl (0.03M Tris-H_2SO_4, 0.3M NaCl, pH 8.3). Five ml of the clarified lysate was applied to the column at a flow rate of 1ml/min. Following the lysate, 5ml of buffer (0.03M Tris-H_2SO_4, 0.3M NaCl, pH 8.3) was passed through the column to flush out unbound protein. A step-wise elution with 0.03M Tris-H_2SO_4 buffer of increasing salt (NaCl) concentrations (.4 M to 1.0M) was carried out and four 1 ml fractions were collected at each buffer concentration. Amicon centriconYM-50 concentrators (Millipore, Billerica, MA) were used to concentrate each fraction.

Gel filtration

Protein fractions positive for bromoperoxidase activity by Phenol Red assay (described below) were pooled and further purified by gel filtration. The gel filteration column contained Superdex^{TM} 200 prep grade resin (Amersham Biosciences, Piscataway, NJ). The column was 0.6 inches in diameter and 6 inches in length. The column was equilibrated with 20 ml of 0.03M Tris-H_2SO_4, 0.4M NaCl buffer, at pH 8.3. 500 µl of the concentrated protein was applied to the column and 1ml fractions were collected. Absorbance at OD_{280} nm was read for all fractions in order to determine the protein containing fractions. Fractions with positive absorbance values at OD_{280} were concentrated as before, using the Amicon centricon-50 concentrators. The presence of bromoperoxidase within these fractions was measured with the Phenol Red assay.

Native PAGE (polyacrylamide gel electrophoresis)

Protein fractions positive for bromoperoxidase were separated on a 10-15% gradient gel, under non-denaturing conditions. The PhastSystem by Amersham

Biosciences (Piscataway, NJ) was employed for gel electrophoresis. Two identical gels were electrophoresed. One gel was stained by coomassie blue, which stained all protein bands present in the fraction. The protein band corresponding to bromoperoxidase activity was identified by activity staining on the second electrophoresed gel. For BPO activity staining, the gel was submerged in Phenol Red assay solution (reagent A) containing 15.6mM hydrogen peroxide and BPO activity was detected by the appearance of a blue colored protein band. Following this, the most purified BPO fraction was separated a second time on a 8-25% gradient gel. Again two gels were run; one for coomassie blue staining and the other for BPO activity staining.

Phenol Red Assay

Bromoperoxidase activity was detected for each concentrated fraction using the Phenol Red assay. The phenol red reagent for bromoperoxidase detection is a mixture of 0.002% phenol red, 1M sodium bromide and 15.6mM hydrogen peroxide (H_2O_2) in a 1M sodium acetate buffer, at pH 5.5 (Reagent N). Non-heme bromoperoxidase activity was detected by adding 10mM sodium azide to the Phenol Red assay reagent mix (Reagent A) [10]. Both, Reagent N and Reagent A are made without hydrogen peroxide, as it is not stable over a long period of time. Hydrogen peroxide is added at the time of the reaction.

In a reaction mixture of 100µl; 90µl of Reagent A or Reagent N, 10µl of concentrated protein and 2µl of 3% hydrogen peroxide are added. The reaction completion is indicated by a yellow to blue color change.

Monochlorodimedone (MCD) Assay

Bromoperoxidase activity for the purified bromoperoxidase protein (dilute and concentrated) was measured by determining the amount of MCD consumed. The BPO activity was measured by the decrease in absorbance at 290nm due to the conversion of monochlorodimedone to bromomonochlorodimedone. The MCD assay reagent consisted of 50 μM MCD, 10 mM hydrogen peroxide, 10 mM Sodium azide, 100mM bromide in a 1M sodium acetate buffer, at pH 5.5. The conversion of one micromole of MCD in one minute by the enzyme is defined as one Unit (U) of enzyme activity [60]. Eventually, all the MCD is consumed and the OD becomes zero. OD of zero indicates reaction termination. The time taken for the complete consumption of 50 μM MCD is used to calculate the amount of enzyme that consumes 1 μM of MCD in 1 minute, which is the specific activity of the enzyme.

RESULTS

Bacteria isolated from the slime layer of the acorn worm

Figure 1 shows a photograph of the acorn worm from which the bacteria were isolated. This is not the entire worm, as it is difficult to pull the whole worm out of the burrow it inhabits. A total of fifty bacterial isolates were obtained from the acorn worm slime and body impressions. The bacteria were grouped on the basis of slant morphology and color. Table 4 shows 16 groups defined for the fifty isolates. Thirty six bacteria were isolated from the slime layer dilutions and 14 from the acorn worm body impressions. They ranged in color from white to yellow to brown. Many isolates had an iodine-like odor, including isolate#4. Isolate #4 is classified in a group by itself and had a thick creamy white, dull slant with irregular edges. Isolate #4 was arbitrarily selected for further study, as it was one of the iodine-like odor releasing bacteria obtained by dilutions of the slime onto Marine agar 2216.

Screening of the isolates for bromoperoxidase activity

Partially purified isolate #4 protein showed BPO activity with phenol red at pH5.5. The BPO activity was identified in the ion exchange concentrated fractions #3 and #4 eluted with 0.4M NaCl (0.03M Tris-H_2SO_4, 0.4M NaCl, pH 8.3) (Table 5). A positive reaction *i.e.* a color change from yellow to blue, with the Phenol Red reagent was observed in less than 2 hours. *Streptomyces aureofaciens* was the positive control for bromination and heat inactivated protein was used as the negative control. The reaction is valid until the heat inactivated protein changes color. The negative control did not

change color in the seven hours the activity assay was incubated. The 0.4M NaCl eluted fraction #3 for isolate #4 was positive for bromoperoxidase activity at 2 hours, whereas fraction #4 showed bromoperoxidase activity in 1 hour. The enzyme activity was not inhibited by the addition of sodium azide, which classifies the bromoperoxidase of isolate #4 as a non-heme bromoperoxidase.

Characterization of isolate #4

Isolate #4 is a Gram positive spore-forming rod. The 16S rDNA amplification using universal bacterial 16S rDNA primers amplified a 1500 base pair band [32]. The sequence of 700 base pairs of this product revealed 99% homology for isolate #4 with *Bacillus cereus* and *Bacillus sp.* 16S ribosomal DNA (Figure 2). Isolate #4 is in the general group of the *Bacillus* genus. It also shows some homology with other Gram positive bacteria such as *Aneurinibacillus sp.*, *Alicyclobacillus sp.*, *Clostridium sp.* and *Streptococcus sp.*

The biochemical tests however, show some very important differences between isolate #4 and *Bacillus sp.* (Table 6). Isolate #4 is catalase negative whereas *B. cereus* and most *Bacillus. sp* are catalase positive. The colony morphology of the isolate resembles the morphology common to *Bacillus sp.*

Growth curve

Bacterial growth over 30 hours in 1liter culture showed an actively growing culture for about 20 hours (Figure 3). The generation time was calculated to be 86min/generation. The lag phase lasted only three hours, followed by an exponential

phase for 16 hours, after which the cells started dying. The bromoperoxidase enzyme was produced in the later part of the growth cycle, starting from 10 hours of growth and lasting up to 28 hours of growth. A strong iodine- like odor was observed during the growth of isolate #4 in liquid culture, in conjunction with bromoperoxidase activity. Enzyme activity stopped at the beginning of spore formation.

Bromoperoxidase purification

Figure 4 shows the BPO protein separated on a 10-15% gradient native gel. Figure 4(a) represents the coomassie blue stained protein gel, whereas Figure 4(b) shows Phenol Red stained BPO activity gel. Both the *S. aureofaciens* protein (Lane 2) and the protein purified from isolate # 4 (Lane 7) show bands less than 67kDa on the 10-15% native gradient gel. These protein bands also reacts with Phenol Red assay reaction mix (Reagent A) at pH 5.5, to produce a blue color band in less than 1 hour.

Protein fraction electrophoresed in Lane 7 (Figure 4) was further analyzed for purity by native PAGE on a 8-25% native gradient gel (Figure 5). Multiple smaller protein bands were observed (Lane 5, Figure 5). The size of the BPO protein was determined to be 62kDa (Figure 5). This was deduced from the gel stained for enzyme activity (Figure 5(b), Lane 7). The two bands below the 62kDa BPO band (Lane 5) could be degradation products or contaminating proteins. Therefore, the BPO protein is only 95% pure at this stage.

Protein purification

The specific activity of the 95% purified protein was approximately 25.58 U/mg protein as shown in Table 7. Partially purified protein obtained by ion exchange chromatography had a specific activity of 0.15 U/mg protein. The specific activity increased to 0.42 U/mg protein when the protein was concentrated after anionic exchange. MCD assay could not be performed on the crude extract (lysate) because of its high turbidity, which interfered with the absorbance reading.

Recovery of the purified protein, after gel filtration and concentration was 11%. 70% of the protein was recovered from concentration after anionic exchange. The total amount of purified protein was 0.043 milligram (mg).

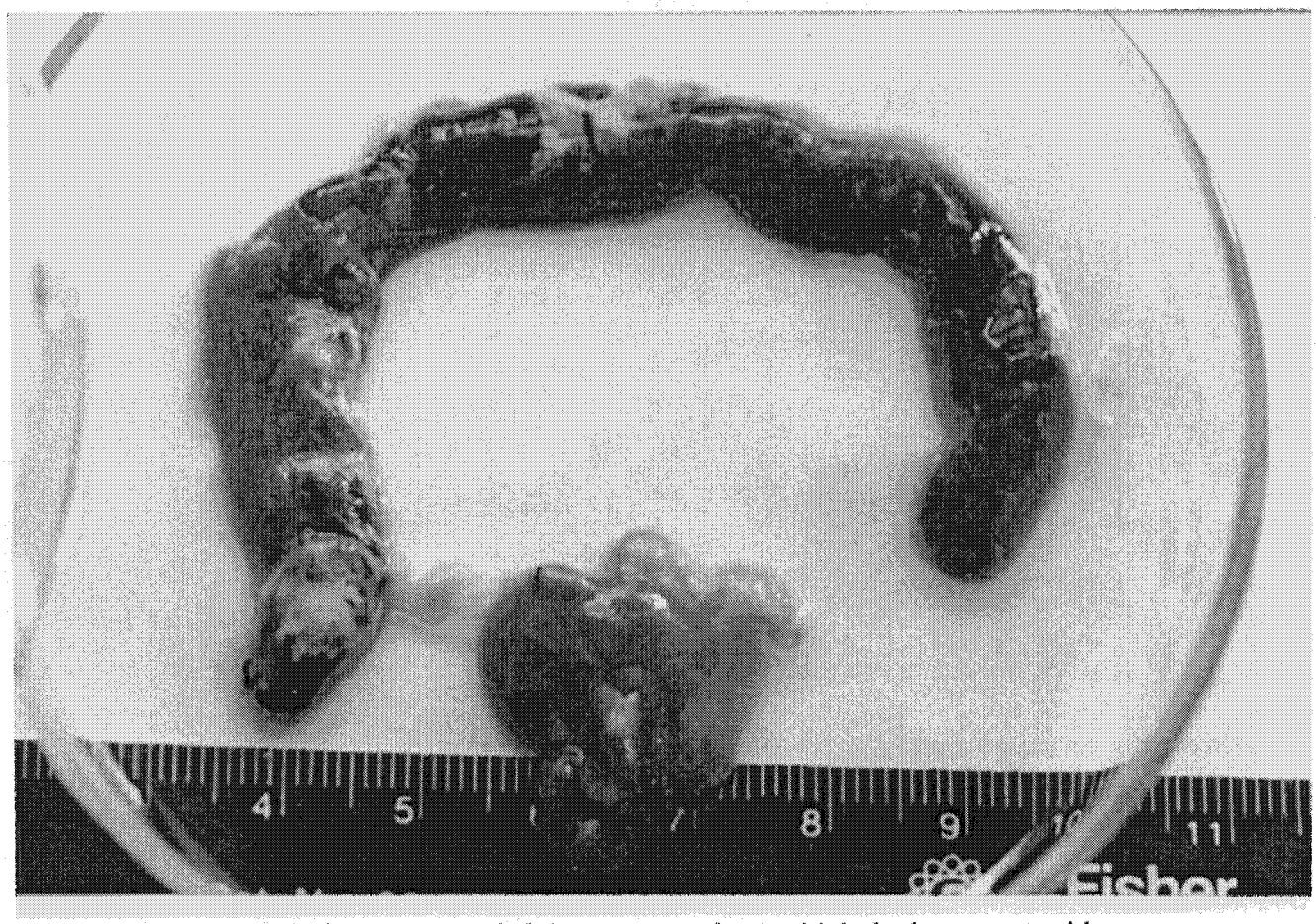


Figure. 1: Photograph of the Red banded Acorn worm from which the bromoperoxidase producing bacterium isolate #4 was isolated.

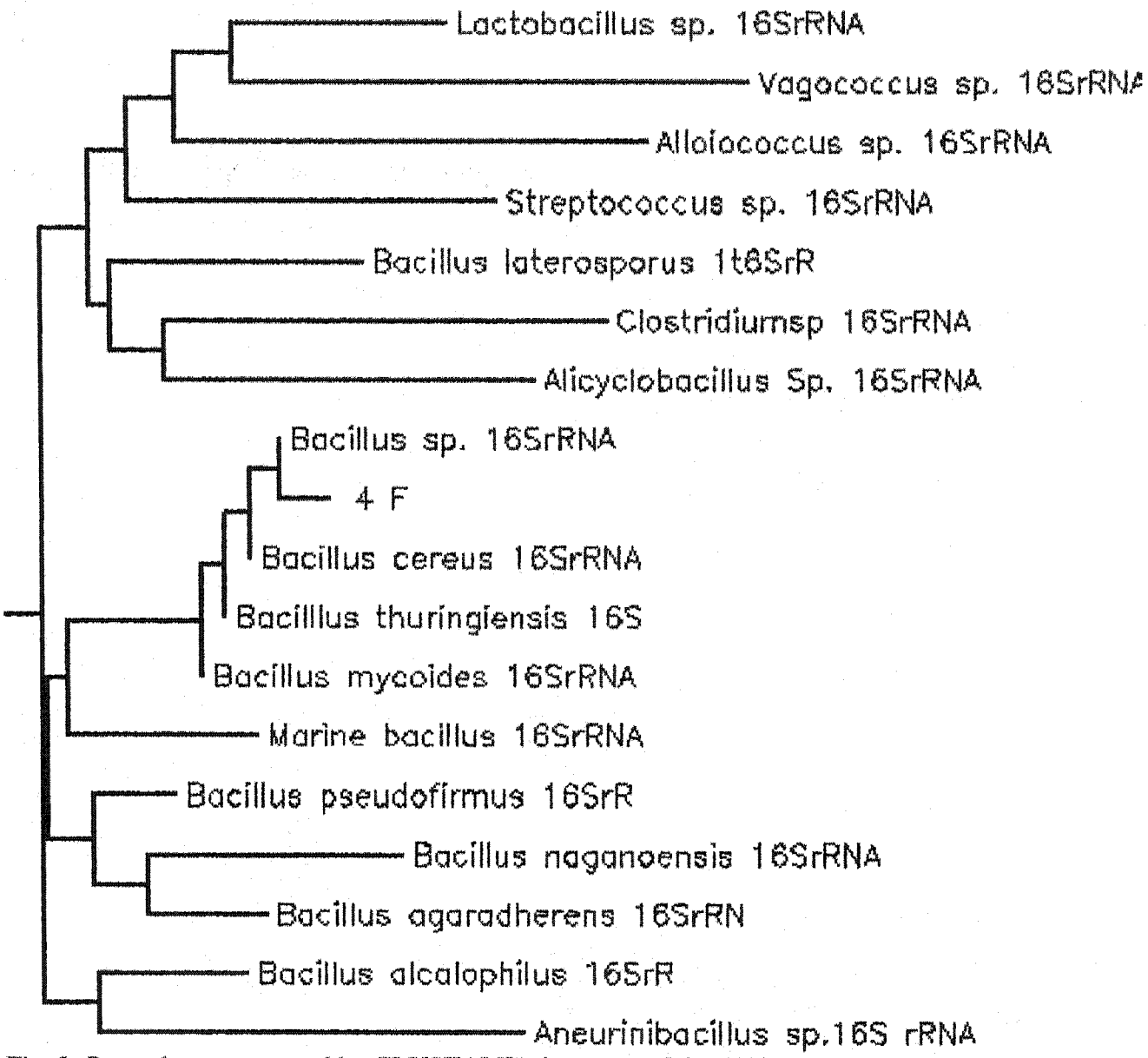


Fig. 2: Rooted tree generated by CLUSTALW alignment of the 700bp rDNA sequence of isolate #4.

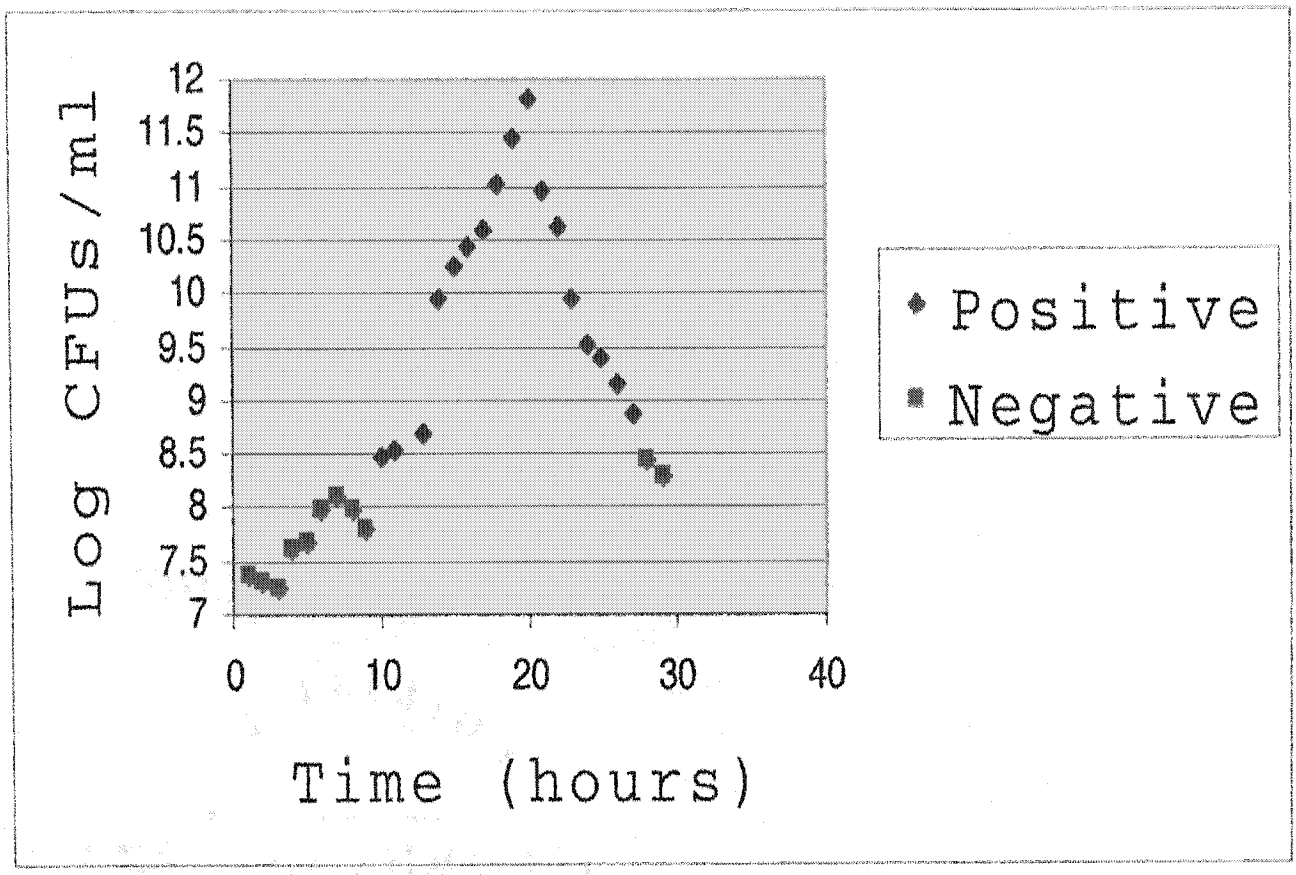


Figure. 3: Bacterial growth curve correlated with the appearance of bromoperoxidase activity. Positive means the presence of bromoperoxidase activity detected by the Phenol Red assay. Negative refers to the absence of bromoperoxidase activity.

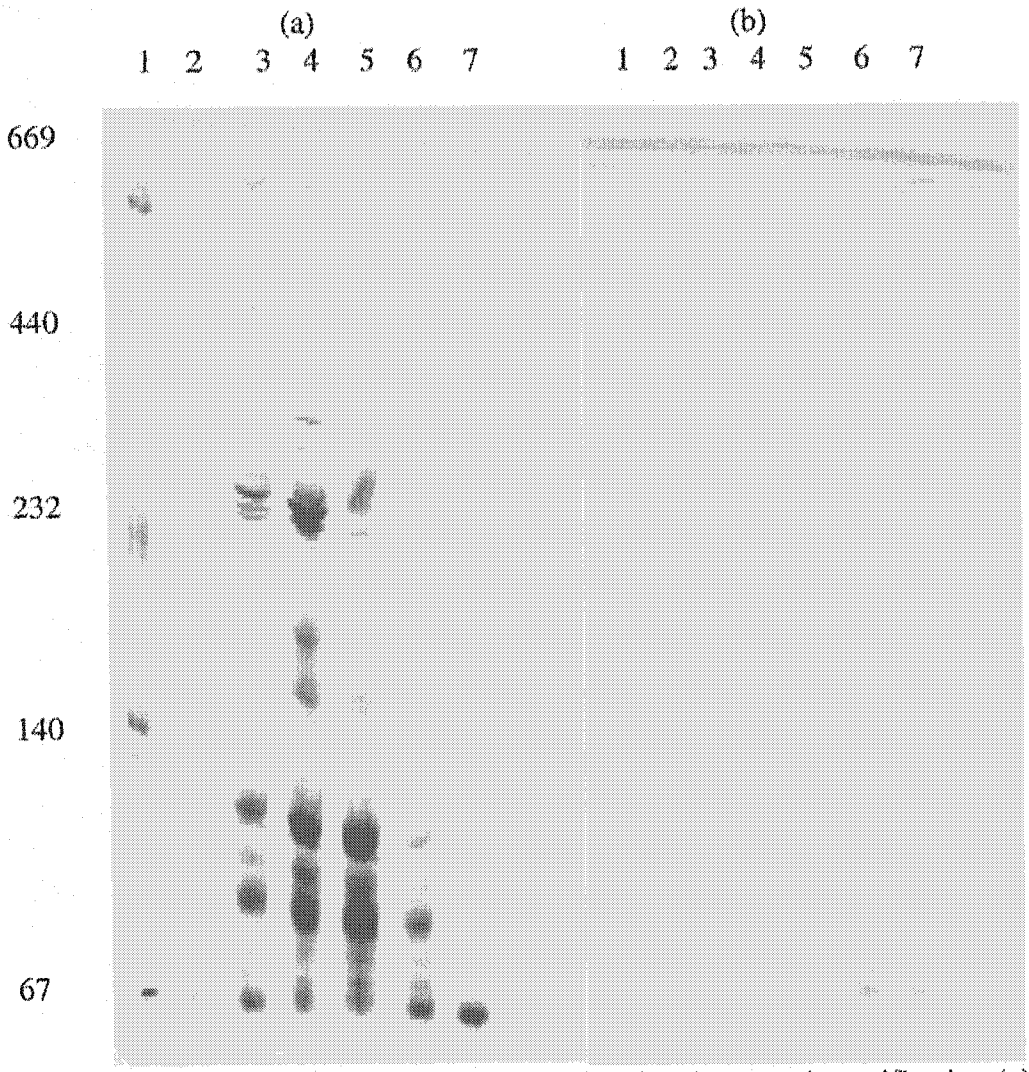


Figure 4: 10-15% gradient native PAGE showing protein purification (a) and in gel enzyme activity of the protein with phenol red (b). Lane 1. High molecular weight native PAGE markers in kDa; lane 2. bromoperoxidase from *Streptomyces aureofaciens*; lane 3. partially purified (ion exchange) bromoperoxidase from Isolate #4; lanes 4, 5, 6,7. bromoperoxidase containing fractions obtained after gel filteration and protein concentration. Lane 7. shows the most purified bromoperoxidase fraction.

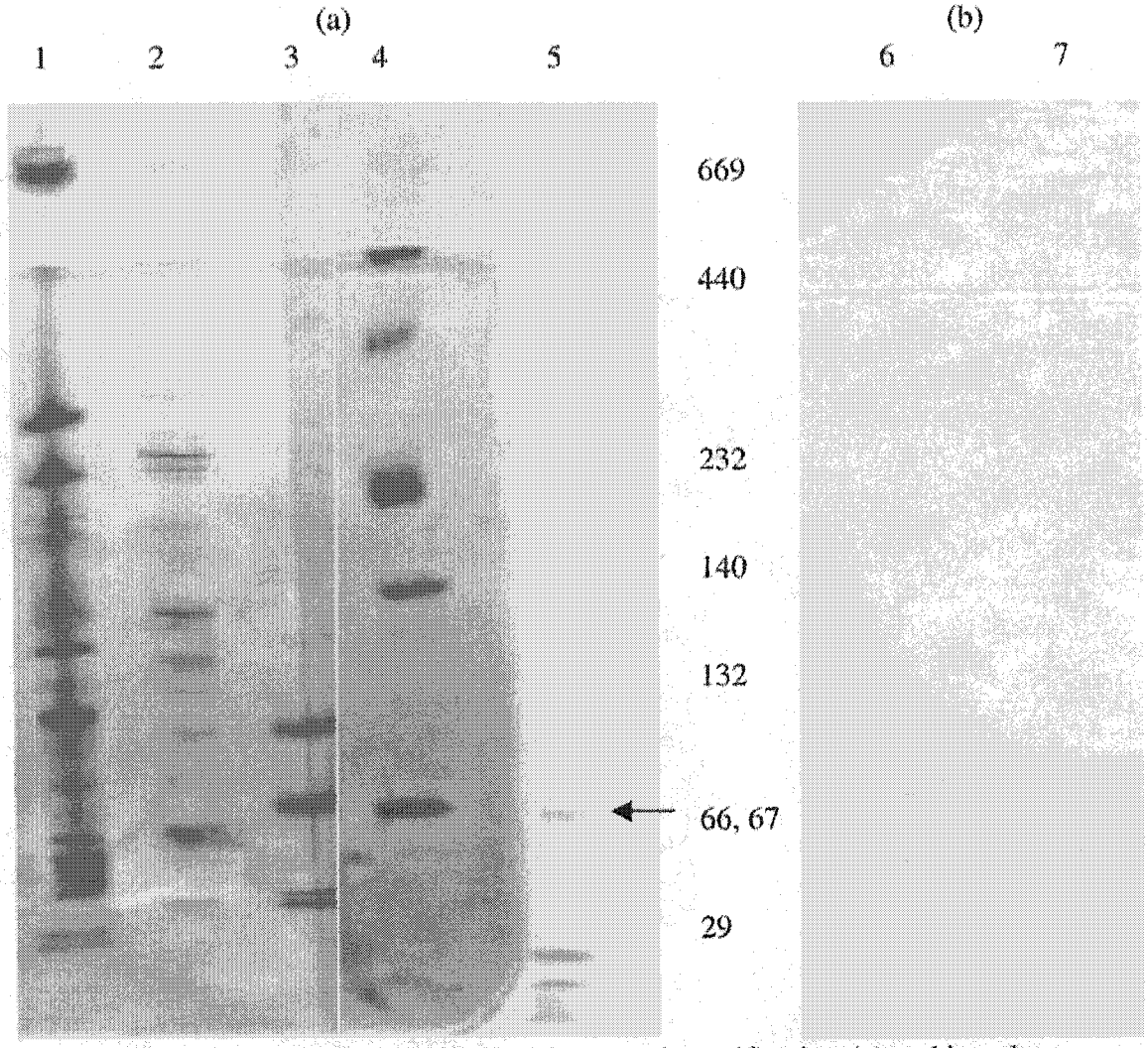


Fig 5: 8-25% gradient native PAGE showing protein purification (a) and in gel enzyme activity of the protein with phenol red (b). Lane 1. partially purified bromoperoxidase of Streptomyces aureofaciens; lane 2. partially purified bromoperoxidase of Isolate #4 (protein after ion exchange); lane 3. low molecular weight native PAGE marker consisting of 132, 66 and 29 kDa protein bands; lane 4. high molecular weight native PAGE marker (kDa); lane 5. bromoperoxidae from Isolate #4 (protein concentrated after gel filteration); lane 6. bromoperoxidase band for *Streptomyces aureofaciens*, shown by activity staining with phenol red; lane 7. phenol red stained bromoperoxidase band from Isolate #4. The arrow indicates the bromoperoxidase protein band.

Table 4: Grouping of the acorn worm bacterial isolates on the basis of slant morphology.

Number of isolates	Isolation method	Group	Group Description based on the agar slant appearance	
			Color	Slant morphology
8	Slime*	A	Light yellow orange	Smooth and shiny; entire edges
1	Slime	B	Medium Yellow orange	Smooth and shiny; entire edges with budges and around bulb at the base of the streak
6	Slime	C	Off-white	Smooth and shiny; entire edges
1	Slime	D	Thick creamy white	Dull; irregular edges (ISOLATE #4)
7	Slime	E	Light yellow orange	Smooth; irregular edges; Bulb at the streak base
9	Slime	F	Creamy white	Smooth and shiny; Beaded; irregular edges
1	Slime	G	Creamy white	Smooth and shiny; entire edges
1	Slime	H	Light brown	Smooth and shiny; entire edges; Bulb at the slant's base
1	Slime	I	Creamy white	Rough texture; oily shine; entire edges
1	Slime	J	Light pink	Smooth and shiny; irregular serrated edges
5	Body#	K	Orange pink	Smooth and shiny; entire edges
1	Body	L	clear	Shiny; irregular edges
2	Body	M	Dark orange	Smooth and shiny; entire edges
2	Body	N	Light yellow orange	Dull; irregular edges
2	Body	O	orange	Smooth and shiny; irregular edges
2	Body	P	white	Flat and dry; entire edges

*Slime dilutions in .1M sterile saline

Acorn worm body impressions

Table 5: Phenol Red Assay on the partially purified protein of isolate #4 and *Streptomyces aureofaciens*. Yellow (Y), yellow brown (Yb), gray (G), gray pink (GP), blue (B). Reagent A contains 0.002% phenol red, 1M sodium bromide, 10mM sodium azide, and 15.6mM hydrogen peroxide in a 1M sodium acetate buffer at pH 5.5. Reagent B contains 0.002% phenol red, 1M sodium bromide, and 15.6mM hydrogen peroxide in 1M sodium acetate buffer, at pH 5.5

			Streptomyces aureofaciens .4 M NaCl				Isolate #4 .4 M NaCl			
	Negative control		Fraction #3		Fraction #4		Fraction #3		Fraction #4	
Time	Reagent A	Reagent N	A	N	A	N	A	N	A	N
1 hour	(-) Y	(-) Y	(-)Y	(+)B	(-)Y	(-)Y	(-)GP	(-)GP	(+)B	(+)B
2 hours	(-) Y	(-) Y	(-)Y	(+)B	(-)Yb	(-)G	(+)B	(+)B	(+)B	(+)B
4 hours	(-) Y	(-) Y	(-)G	(+)B	(-)G	(+)B	(+)B	(+)B	(+)B	(+)B
6 hours	(-) Yb	(-) Yb	(+)B	(+)B	(+)B	(+)B	(+)B	(+)B	(+)B	(+)B
7 hours	(-) Yb	(-) Yb	(+)B	(+)B	(+)B	(+)B	(+)B	(+)B	(+)B	(+)B

Table 6: Biochemical tests for the Acorn worm associated bacterium (isolate #4)

Characteristics	**Isolate #4**	***B.cereus***
Colony morphology	Round, thick, opaque	Round, thick, opaque
Colony color	Cream	Cream
Cell shape	Rod	Rod
Gram stain	Gram positive	Gram positive
Spore stain	Positive	Positive
Flagella stain	Positive	Positive
Oxidase	Positive	Positive
Catalase	Negative	Positive
Indole	Negative	NA
Arginine	Positive	NA
Nitrate	Positive	Positive
Gelation	Positive	Positive
Glucose	Positive	Positive
Citrate	Positive	Positive

Table 7: Protein purification table for the bromoperoxidase enzyme isolated from isolate #4.

Purification step	Volume (ml)	Protein (mg/ml)	Total Protein (mg)	Total activity (U)	Specific Activity (U/mg protein)	Fold purification	Recovery (%)
Crude extract	30	2.8	86	-	-	-	-
HiTrap Q HP column*	6	0.9	5.4	0.83	0.15	1	100
Amicon centricon-50♣	2	1.9	3.8	1.6	0.42	2.8	70.37
Gel filteration♦ and concentration	0.03	1.43	.043	1.1	25.58	170.53	11.3

*Sepharose column by Amersham Biosciences

♣Used to concentrate protein greater than 50kDa

♦Sephadex 200 resin

DISCUSSION

Many halogenated compounds have been isolated from the habitat (burrows) of acorn worms. However, the presence of bromoperoxidase producing bacteria associated with the acorn worm and its environment has not been explored. We have shown the presence of a large bacterial population associated with the Red-Banded acorn worm from the Florida coast. We isolated fifty different strains from the slime associated with this acorn worm and the acorn worm body impressions. Most strains varied in their colony morphology and displayed a wide range of pigmentation. Many had iodine-like odor. We have initiated the screening of these isolates and at present about 50% seem to have bromoperoxidase activity. This makes the marine acorn worm an important environment to be explored for bromoperoxidases.

This is the first report for the isolation of a bromoperoxidase producing bacterium from an acorn worm slime layer, although a number of organohalogen compounds from the acorn worm and its environment have been studied. Isolate #4 was identified as a Gram positive rod, obtained from the slime layer of the Red-Banded acorn worm. BLAST and phylogenetic analysis on the 700 base pairs of the sequenced rDNA showed 99% homology to the *Bacillus* genus, specifically to *B. cereus*. The isolate differs from *Bacillus* by being catalase negative. All known *Bacillus sp.* are catalase positive. Isolate #4 presumptively belongs to the *Bacillus* genus but maybe an unknown species. In general the 16S rDNA sequences of all *Bacillus sp.* were very similar and they also shared close homology with the 16SrDNA sequences of other Gram positive rods. Isolate #4's rDNA sequence was most closely related to *B. cereus*. This close homology

of the rDNA is attributed to the highly conserved nature of the ribosomal DNA. The entire 16S rDNA gene needs to be sequenced to identify the proper placement of isolate #4. In addition, a better homology comparison would be to amplify several regions of isolate #4 apart from the rDNA regions and align these sequences with other *Bacillus* species.

Isolate #4 grows optimally at room temperature in a medium containing large amounts of salt (20 grams in 1 liter). It has a long growth period of about 24 to 30 hours before the production of spores. The generation time is 86min. Bromoperoxidase enzyme activity is seen in the later stages of growth. Therefore, the enzyme seems to be involved in the production of secondary metabolites.

Partial purification of the bromoperoxidase protein was accomplished by anion exchange chromatography, as the bromoperoxidase protein is a negatively charged protein at pH 8.2. A large number of proteins, including the BPO protein were obtained by anionic exchange. Fractions from anionic exchange chromatography had to be concentrated for enzyme activity measurements. The Phenol Red Assay showed enzyme activity in acidic pH of 5.5 and the enzyme activity was not inhibited by other interfering proteins. The enzyme also showed activity in the presence of sodium azide in the reaction mix (Reagent A), which makes it a non-heme bromoperoxidase. However, structural studies on the protein are required to detect the presence or absence of other metal ions.

The 95% purified enzyme has a molecular weight of approximately 62kDa. as determined by activity staining. We did not completely purify the BPO protein as two

extra low molecular weight bands were observed on 8-25% native gradient gel. These bands might be degraded BPO protein or low MW proteins that were not removed. Repeat of gel filteration using FPLC (fast performance liquid chromatography) as well as addition of protease inhibitors maybe required for the complete BPO protein purification.

Our future studies will focus on the complete purification of the protein and the study of its reaction mechanism using various substrates. N-terminal sequencing of the purified protein will be performed. This will enable us to generate primers to the bromoperoxidase gene and to isolate the gene from the chromosome. The BPO gene can then be cloned and overexpressed in a bacterial system such as *Escherichia coli*, to obtain large quantities of the BPO protein. The BPO protein sequence will also enable us to do comparative studies on the BPO from isolate # 4 with other non-heme bromoperoxidases.

The enzyme's halogenation reaction mechanism and protein structure is also of interest. Examination of the structure of the enzyme will elucidate if the reaction mechanism is similar to other enzymes in this group. This will involve studying the halogenated products obtained using different substrates for halogenation by the BPO of isolate # 4. The halogenated products generated are expected to vary with the substrate used. Variation of reaction conditions will allow us to determine the enzymes range of halometabolites produced and assist in enzyme specificity determination and the enzyme's potential use in the industry as a catalyst for halogenation and oxidation reactions.

Identification of additional halogenation enzymes, especially halogenases from isolate # 4, is another aspect of future studies. This will involve designing halogenase

primers with the aid of currently known halogenase genes. PCR on the genomic DNA of isolate # 4 utilizing these primers will help in identifying any halogenase gene present. Identification of halogenase gene (present or absent) in conjunction with studying the reaction mechanism of the BPO from isolate # 4 will address the recent findings whether halogenases are more specific and mostly involved in the production of halogenated compounds.

In conclusion, we have isolated a Gram positive, non-heme bromoperoxidase producing *Bacillus* from the slime layer of the red-banded acorn worm from the Florida coast. We have also purified the 62kDa BPO enzyme to 95% homogeneity and determined the specific activity of this enzyme. After complete purification of the enzyme the reaction mechanism will be studied. In addition, we will identify the bacterium and determine the presence of additional halogenating enzymes such as halogenases in isolate # 4.

Acknowledgements

We thank Dr. P. Grilione and Dr. Okuda for their advice.

Funding for this project was partly provided by NSF grant 94-79, RUI Program.

REFERENCES

1. Ahern TJ, Allan GG, Medcalf DG (1980) New bromoperoxidases of marine origin: Partial purification and characterization. Biochimica et Biophysica Acta 616(2):329-339

2. Almeida M, Humanes M, Melo R, Silva JA, Fraústo da Silva JJR, Vilter H, Wever R (1998) *Saccorhiza polyschides (Phaeophyceae; Phyllariaceae)* A new source for vanadium-dependent haloperoxidases. Phytochemistry 48(2):229-239

3. Almeida MG, Humanes M, Melo R, Silva JA, Fraústo da Silva JJRF, Wever R (2000) Purification and characterization of vanadium haloperoxidases from the brown alga *Pelvetia canaliculata*. Phytochemistry 54:5-11

4. Almeida M, Filipe S, Humanes M, Maia MF, Melo R, Severino N, da Silva JAL, Fraústo da Silva JJRF, Wever R (2001) Vanadium haloperoxidases from brown algae of the Laminariaceae family. Phytochemistry (Oxford) 57(5):633-64

5. Andersson MA, Allenmark SG (1998). Asymmetric sulfoxidation catalyzed by a vanadium bromoperoxidase: Substrate requirements of the catalyst. Tetrahedron 54:15293-15304

6. Ashworth RB, Cormier MJ (1967) Isolation of 2,6-Dibromophenol from the marine hemichordate, *Balanoglossus biminiensis*. Science 155:1558-1559

7. Baden DG, Corbett MD (1980) Bromoperoxidases from *Penicillus capitatus*, *Penicillus lamourouxii* and *Rhipocephalus phoenix*. Journal of Biochemistry 187:205-211

8. Bantleon R, Altenbuchner J, van Pee KH (1994) Chloroperoxidase from *Streptomyces lividans*: Isolation and characterization of the enzyme and the corresponding gene. J. Bact 176(8):2339-2347

9. Bar-Nun, N, Shcolnick S, Mayer AM (2002) Presence of a vanadium-dependent haloperoxidase in *Botrytis cinerea*. FEMS Microbiology Letters 217:121-124

10. Butler A, Walker JV (1993) Marine Haloperoxidases 93:1937-1944

11. Butler A (1998) Vanadium haloperoxidase. Current Opinion in Chemical Biology 2:279-285

12. Butler A (1999) Mechanistic considerations of the vanadium haloperoxidases. Coordination Chemistry Reviews 187:17-35

13. Carter JN, Beatty KE, Simpson MT, Butler A (2002) Reactivity of recombinant and mutant vanadium bromoperoxidase from the red alga *Corallina Officinalis*. Journal of Inorganic Biochemistry 91:59-69

14. Carter-Franklin JN, Parrish JD, Tschirret-Guth RA, Little RD, Butler A (2003) Vanadium haloperoxidase-catalyzed bromination and cyclization of terpenes. Journal of American Chemical Society 125:3688-3689

15. Căsný M, Rehder D, Schmidt H, Vilter H, Conte V (2000) A ^{17}O NMR study of peroxide binding to the active centre of bromoperoxidase from *Ascophyllum nodosum*. Journal of Inorganic Biochemistry 80:157-160

16. Chen YP, Lincoln DE, Woodin SA, Lovell CR (1991) Purification and properties of a unique flavin-containing chloroperoxidase from the capitellid polychaete *Notomastus lobatus*. Journal of Biological Chemistry 266(35):23909-23915

17. Conte V, Furia FD, Moro S (1996) Synthesis of brominated compounds. A convenient molybdenum-catalyzed procedure inspired by the mode of action of haloperoxidases. Tetrahedron Letters 37(47):8609-8612

18. Corgiat JM, Dobbs FC, Burger MW, Scheuer PJ (1993) Organohalogen constituents of the acorn worm *Ptychodera bahamensis*. Comp. Biochem. Physiol 106B:83-86

19. de Boer E, Tromp MGM, Plat H, Krenn GE, Wever R (1986) Vanadium-V as an essential element for haloperoxidase activity in marine brown algae *Laminaria saccharina* purification and characterization of a vanadium-V containing bromoperoxidase from *Laminaria saccharina*. Biochimica et Biophysica Acta 872(1-2):104-115

20. de Boer E, Van K, Troop MG, Plat H, Wever R (1986) Bromoperoxidase from *Ascophyllum nodosum*: a novel class of enzymes containing vanadium as a prosthetic group. Biochimica et Biophysica Acta 869:48-53

21. de Boer E, Keijzers CP, Klaassen AAK, Reijerse EJ, Collison D, Garner CD, Wever R (1988) 14N-coordination to VO2+ in reduced vanadium bromoperoxidase, an electron spin echo study. FEB 235:93-97

22. de Boer E, Wever R (1988) The reaction mechanism of the novel vanadium bromoperoxidase. J Biol Chem 263:12326-12332

23. de Schrijver A, Nagy I, Schoofs G, Proost P, Vanderleyden J, van Pee KH, de Mot R (1997) Thiocarbamate herbicide-inducible nonheme haloperoxidase of *Rhodococcus erythropolis* NI86/21. Applied and Environmental Microbiology 63(5): 1911-1916

24. Facey SJ, Grob F, Vining VC, Yang K, and van Pee KH (1996) Cloning, sequencing and disruption of a bromoperoxidase-catalase gene in *Streptomyces venezuelae*: evidence that it is not required for chlorination in chloramphenicol biosynthesis. Microbiology 142:657-665

25. Fenna RE (1987) Crystallization and subunit structure of canine myeloperoxidase. J Mol Biol 196:919-925

26. Findlay JA, Patil AD (1986) Antibacterial constituents of the red alga *Cystoclonium pupureum*. Phytochemistry (Oxford) 25(2):548-550

27. Franssen MCR, Posthumus MA, van der Plas HC (1988) Halometabolites from *Caldariomyces fumago*. Phytochemistry (Oxford) 27(4):1093-1096

28. Franssen MCR, van der Plas HC (1992) Haloperoxidases: their properties and their use in organic synthesis. Advances in Applied Microbiology 37:41-99

29. Giray C, King GM (1997) Predator deterrence and 2,4-dibromophenol conservation by the enteropneusts *Saccoglossus bromophenolosus* and *Protoglossus graveolens*. Marine Ecology-Progress Series 159:229-238

30. Hager LP, Morris DR, Brown FS, Eberwein H (1996) Chloroperoxidase II. Utilization of halogen anions. Journal of Biological Chemistry 241:1769-1777

31. Hara I, Sakurai T (1998) Isolation and characterization of vanadium bromoperoxidase from a marine macroalga, *Ecklonia stolonifera*. Journal of Inorganic Biochemistry 72(1-2):23-28

32. Hewson WD, Hager LP (1980) Bromoperoxidase and halogenated lipids in marine alga. J Phycol 67:340-345

33. Higa T, Scheuer PJ (1975) 3-Chloroindole, principal odorous constituent of the hemichordate *Ptychodera flava laysanica*. Naturwissenschaften 62:395-396

34. Higa T, Fujiyama T, Scheuer PJ (1980) Halogenated phenol and indole constituents of acorn worms. Comp Biochem Physiol 65B:525-530

35. Isupov MN, Dalby AR, Brindley AA, Izumi Y, Tanabe T, Murshudov GN, Littlechild JA (2000) Crystal structure of dodecameric vanadium-dependent bromoperoxidase from red algae *Corallina officinalis* 299:1035-1049

36. Itoh N, Izumi Y, Yamada H (1985) Purification of bromoperoxidase from *Carollina pilulifera*. Biochem Biophy Res Comm 131:428-435

37. Itoh N, Morinaga N, Nomura A (1992) A variety of catalases and bromoperoxidases in genus Pseudomonas and their characterization. Biochimica et Biophysica Acta 1122:189-195

38. Itoh N, Morinaga N, Kouzai T (1993) Oxidation of aniline to nitrobenzene by nonheme bromoperoxidase. Biochemistry and Molecular Biology Int 29(4):785-791

39. Itoh N, Morinaga N, Kouzai T (1994) Purification and characterization of a novel metal containing nonheme bromoperoxidase from *Pseudomonas putida*. Biochimica et Biophysica Acta 1207:208-216

40. Itoh N, Sasaki H, Ohsawa H, Shibata MS, Miura J (1996) Bromoperoxidase in *Corallina pilulifera* is regulated by its vanadate content 42(2):277-281

41. Itoh N, Kawanami T, Liu JQ, Dairi T, Miyakoshi M, Nitta C, Kimoto Y (2001) Cloning and biochemical characterization of cobalt activated bromoperoxidase-esterase from *Pseudomonas putida* IF-3 strain. Biochimica et Biophysica Acta 1545:53-66

42. Jakopitsch C, Regelsberger G, Furtmüller PG, Rüker F, Peschek GA, Obinger C (2001) Catalase-peroxidase from *Synechocystic* is capable of chlorination and bromination reactions. Biochemical and Biophysical Research Communications 287:682-687

43. Jordan P, Kloareg B, Vilter H (1991) Detection of vanadate-dependent bromoperoxidases in protoplasts from the brown algae *Laminaria digitata* and *Laminaria saccharina*. Journal of Plant Physiology 137(5):520-524

44. Kataoka M, Honda K, Shimizu S (2000) 3,4-Dihydrocoumarin hydrolase with haloperoxidase activity from *Acinetobacter calcoaceticus* F46. European Journal of Biochemistry 267(1):3-10

45. Kawanami T, Miyakoshi M, Dairi T, Itoh N (2002) Reaction mechanism of the CO2+- activated multifunctional bromoperoxidase-esterase from *Pseudomonas putida* IF-3. Archives of Biochemistry and Biophysics 398(1):94-100

46. King GM (1986) Inhibition of microbial activity in marine sediments by a bromophenol from a hemichordate. Nature 323:257-259

47. Kirner S, Krauss S, Sury G, Lam ST, Ligon JM, van Pee KH (1996) The non haem chloroperoxidase from *Pseudomonas flourescens* and ite relationship to pyrrolnitrin biosynthesis. Microbiology 142:2129-2135

48. Knoch M, VanPee KH, Vining LC, Lingens F (1989) Purification, properties and immunological detection of a bromoperoxidase-catalase from *Streptomyces venezuelae* and from a chloramphenicol nonproducing mutant. J Gen Micr 135:2493-2502

49. Kraan S, Guiry MD (2000) Molecular and morphological character inheritance in hybrids of *Alaria esculenta* and *A. praelonga* (*Alariaceae, Phaeophyceae*). Phycologia 39(6):554-559

50. Krenn BE, Henk P, Wever R (1988) Purification and characterization of a non-haem bromoperoxidase from *Streptomyces aureofaciens*. Biochimica et Biophysica Acta 952:255-260

51. Krenn BE, Izumi Y, Yamad AH, Wever R (1989) A comparision of different vanadium bromoperoxidase from *Corallina pilulifera* is also a vanadium enzyme. Biochimica et Biophysica Acta 998(1):63-68

52. Kushner DJ (1978) Microbial life in extreme environments. Academic. New York p315-367

53. Küsthardt U, Hedman B, Hodgson KO, Hahn R, Vilter H (1993) High-resolution XANES studies on vanadium-containing haloperoxidase: pH-dependence and substrate binding. Federation of European Biochemical Societies 329:5-8

54. Kuusk H, Björklund M, Rydström J (2001) Purification and characterization of a novel bromoperoxidase-catalase isolated from bacteria found in recycled pulp white water. Enzyme and Microbial Technology 28:617-624

55. Littlechild J (1999) Haloperoxidases and their role in biotransformation reactions. Current Opinion in Chemical Biology 3:28-34

56. Madigan MT, Martinko JM, Parker J (1997) In Brock biology of microorganisms. 8th ed. Prentice Hall, Upper Saddle River, NJ

57. Marshall GC, Wright GD (1996) Purification and characterization of two haloperoxidases from the glycopeptide antibiotic producer *Streptomyces toyocaensis* NRRL 15009. Biochemical and Biophysical Research Communications 219:580-583

58. Martinez JS, Carroll GL, Tschirret-Guth RA, Altenhoff G, Little RD, Butler A (2001) On the regiospecificity of vanadium bromoperoxidase 123:3289-3294

59. Moore CA, Okuda RK (1996) Bromoperoxidase activity in 94 species of marine algae. Journal of Natural Toxins 5(3):295-298

60. Neidleman S, Geigert J (1986) Biological halogenation: principles, basic roles and applications. Ellis Horwood Ltd. Chichester. England

61. Neidleman SL, Geigert J (1987) Biological halogenation: roles in nature, potential in industry. Endeavour 11(1):5-15

62. Nieder M, Hager L (1985) Conversion of α-amino acids and peptides to nitriles and aldehydes by bromoperoxidase. Archives of Biochemistry and Biophysics 240(1):121-127

63. Ohsawa N, Ogata Y, Okada N, Itoh N (2001) Physiological function of bromoperoxidase in the red marine alga, *Corallina pilulifera*: production of bromoform as an allelochemical and the simultaneous elimination of hydrogen peroxide. Phytochemistry 58:683-692

64. Ohshiro T, Nakano S, Takahashi Y, Suzuki M, Izumi Y (1999) Occurrence of bromoperoxidase in the marine green macro alga, *Ulvella lens* and emission of volatile brominated methane by the enzyme. Phytochemistry 52:1211-1215

65. Ohshiro T, Hemrika W, Aibara T, Wever R, Izumi Y (2002) Expression of the vanadium-dependent bromoperoxidase gene from a marine-alga *Corallina pilulifera* in *Saccharomyces cerevisiae* and characterization of the recombinant enzyme. Phytochemistry 60:595-601

66. Olsen RL, Little C (1984) Purification and some properties of myeloperoxidase and eosinophil peroxidase from human blood. Biochem J 209:781-787

67. Pelletier I, Pfeifer O, Altenbuchner J, vanPee KH (1994) Cloning of a second non-heme bromoperoxidase gene from *Streptomyces aureofaciens* ATCC1072: sequence analysis, expression in *S. lividans* and enzyme purification. Microbiology 140:509-516

68. Pelletier I, Altenbuchner J, Mattes R (1995) A catalytic triad is required by the non-heme haloperoxidases to perform halogenation. Biochimica et Biophysica Acta 1250:149-157

69. Pfeifer O, Pelletier I, Altenbuchner J, van Pee KH (1992) Molecular cloning and sequencing of a non-haem bromoperoxidase gene from *Streptomyces aureofaciens* ATCC 10762. Journal of General Microbiology 138:1123-1131

70. Piraee M, Vining LC (2002) Use of degenerate primers and touchdown PCR to amplify a halogeanse gene fragment from *Streptomyces venezuelae* ISP5230. Journal of Industrial Microbiology and Biotechnology 29:1-5

71. Preobrazhenskaya YV, Voskoboev AI, Burd VN (2003) Phosphatase activity of non-heme chloroperoxidase from the bacterium *Serratia marcescens*. FEBS Letters 536(1-3):41-44

72. Renirie R, Wieger H, Wever R (2000).. Peroxidase and phosphatase activity of active-site mutants of vanadium chloroperoxidase from the fungus *Curvularia inaequalis*. Implications for the catalytic mechanisms. Journal of Biological Chemistry 275(16):11650-11657

73. Rorrer GL, Tucker MP, Cheney DP, Maliakal S (2001) Bromoperoxidase activity in microplantlet suspension cultures of the macrophytic red alga *Ochtodes secundiramea*. Biotechnology & Bioengineering 74(5):389-395

74. Rush C, Willetts A, Davies G, Dauter Z, Watson H, Littlechild J (1995) Purification, crystallization and preliminary X-ray analysis of the vanadium-dependent haloperoxidase from *Corallina officinalis*. FEBS Letters 359:244-246

75. Sakurai H, Tsuchiya K A biomimetic model for vanadium-containing bromoperoxidase. FEB 260:109-112

76. Shang M, Okuda RK, Worthen D (1994) Bromination of phenols using an algal bromoperoxidase. Phytochemistry (Oxford) 37(2):307-310

77. Sheffield DJ, Harry TR, Smith AJ, Rogers LJ (1995) *Corallina officinalis* bromoperoxidase immobilized on agarose. Phytochemistry (Oxford) 38(5):1103-1107

78. Shimonishi M, Shinsuke K, Inoue H, Wever R, Ohshiro T, Izumi Y, Tanabe T (1998) Cloning and expression of the gene for a vanadium dependent bromoperoxidase from a marine alga, *Carollina pilulifera*. FEBS letters 428:105-110

79. Smith TS, Pecoraro VL (2002) Oxidation of organic sulfides by vanadium haloperoxidase model complexes. Inorganic Chemistry 41(25):6754-6760

80. Sobek H, Haag T, Pfeifer O, Schomburg D, Lingens F, vanPee KH (1991) Crystallization and Preliminary X-ray data of bromoperoxidase from *Streptomyces aureofaciens* ATCC 10762. J. Mol. Biol 221:35-37

81. Soedjak HS, Butler A (1990) Characterization of vanadium bromoperoxidase from *Macrocystis* and Fucus reactivity of vanadium bromoperoxidase toward acyl and alkyl peroxidases and bromination of amines. Biochemistry 29(34):7974-7981

82. Steward CC, Pinckney J, Piceno Y, Lovell CR (1992) Bacterial numbers and activity, microalgal biomass and productivity, and meiofaunal distribution in

sediments naturally contaminated with biogenic bromophenols. Marine Ecology-Progress Series 90(1):61-71

83. Steward CC, Dixon TC, Chen YP, Lovell CR (1995) Enrichment and isolation of a reductively debrominating bacterium from the burrow of a bromometabolite-producing marine hemichordate. Canadian Journal of Microbiology 41:637-642

84. Steward CC, Nold SC, Ringelberg DB, White DC, Lovell CR (1996) Microbial biomass and community structures in the burrows of bromophenol producing and non-producing marine worms and surrounding sediments. Mar Ecol Prog Ser 133:149-165

85. Tanaka N, Dumay V, Liao Q, Lange AJ, Wever R (2002) Bromoperoxidase activity of vanadate-substituted acid phosphatases from *Shigella flexneri* and *Salmonella enterica ser. typhimurium*. European Journal of Biochemistry 269(8):2162-2167

86. ten Brink HB, Schoemaker HE, Wever R (2001) Sulfoxidation mechanism of vanadium bromoperoxidase from *Ascophyllum nodosum*. Evidence for direct oxygen transfer catalysis. European Journal of Biochemistry 268(1):132-138

87. Terron MC, Verhagen FJM, Franssen MCR, Field JA (1997) Chemical bromination of phenol red by hydrogen peroxidase is possible in the absence of haloperoxidases. Chemosphere 36(6):1445-1452

88. van Pee KH, Lingens F (1984) Detection of a bromoperoxidase in *Streptomyces phaeochromogenes*. FEBS letters 173(1):5-8

89. van Pee KH (1988) Molecular cloning and high level expression of a bromoperoxidase gene from *Streptomyces aureofaciens* Tu24. Journal of Bacteriology 12:5890-5804

90. van Pee KH (2001) Microbial biosynthesis of halometabolites. Archives of Microbiology 175(4):250-258

91. vanPee KH, Unversucht S (2003) Biological dehalogenation and halogenation reactions. Chemosphere 2-13

92. Verdel EF, Kline PC, Wani S, Woods AE (2000) Purification and partial characterization of haloperoxidase from fresh water algae *Cladophora glomerata*. Comparative Biochemistry and Physiology Part B 125:179-187

93. Weisner W, vanPee KH, Lingens F (1986) Detection of a new chloroperoxidase in *Pseudomonas pyrrocinia*. FEBS letters 209:321-324

94. Weng M, Pfeifer O, Krauss S, Lingens F, van Pee KH (1991) Purification, characterization and comparison of two non-haem bromoperoxidases from *Streptomyces aureofaciens* ATCC 10762. J Gen Micr 137:2539-2546

95. Weyand M, Hecht HJ, Kieβ M, Liaud MF, Vilter H, Schomburg D (1999) X-ray structure determination of a vanadium-dependent haloperoxidase from *Ascophyllum nodosum* at 2.0 Å resolution. Journal of Molecular Biology 293: 595-611

96. Woodin SA, Walla MD, Lincoln DE (1987) Occurrence of brominated compounds in soft-bottom benthic organisms. J Exp Mar Biol Ecol 107:209-217

97. Williams & Wilkins. Marine Medium 2216. In J. T. Stanely, M.P. Bryant, N. Pfening and J.G. Holt. Bergey's manual of systematic bacteriology. Vol 3.

98. Zeiner R, vanPee KH, Lingens F (1988) Purification and partial characterization of multiple bromoperoxidases from *Streptomyces griseus*. J Gen Micr 134:3141-3149