ANALYSIS OF ENVIRONMENTAL BACTERIA CAPABLE OF UTILIZING REDUCED PHOSPHORUS COMPOUNDS

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in

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by

Brandee L. Stone

Summer 2011

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P	Phosphorus. Refers to any molecule or compound containing phosphorus.
Pi	Phosphate. Inorganic phosphorus compound. Oxidation state +5.
Hpt	Hypophosphite. Inorganic reduced phosphorus compound. Oxidation state +1.
Pt	Phosphite. Inorganic reduced phosphorus compound. Oxidation state +3.
AEpn	Aminoethylphosphonate. Organic reduced phosphorus compound. Oxidation state +3.
PH_3	Phosphine. Fully reduced phosphorus compound. Oxidation state -3.
htxA	Gene encoding a 2-oxoglutarate-dependent hypophosphite dioxygenase. Responsible for the oxidation of hypophosphite to phosphite.
ptxD	Gene encoding an NAD-dependent phosphite dehydrogenase. Responsible for the oxidation of phosphite to phosphate.
RPO	Reduced phosphorus oxidation
RPOB	Reduced phosphorus oxidizing bacteria

ABSTRACT

ANALYSIS OF ENVIRONMENTAL BACTERIA CAPABLE

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Although bacterial metabolic pathways to oxidize the reduced phosphorus compounds hypophosphite and phosphite have been characterized, bacterial reduced phosphorus oxidation in the environment and the impact of this activity on P biogeochemistry has largely been overlooked. In this study, I attempted to answer two of the basic questions in this field: (1) how abundant are culturable reduced phosphorus oxidizing bacteria in a variety of soils and sediments; and (2) how common are previously characterized bacterial pathways to oxidize hypophosphite and phosphite. To determine how common the ability is in the environment to oxidize hypophosphite and phosphite, I used a 5-tube most probable number method to estimate the concentrations of viable hypophosphite and phosphite oxidizing bacteria from 12 natural aquatic and terrestrial environments in northern California. The percent of total culturable bacterial

concentrations able to use these reduced phosphorus compounds as a sole source of phosphorus were: hypophosphite, 7-100%; phosphite, 10-67%; and aminoethylphosphonate, 34-270%. Relatively high concentrations of reduced phosphorus oxidizing bacteria were found in both pristine sites and sites with urban and agricultural disturbance, and did not correlate with likely concentrations of reduced phosphorus compounds. Concentrations of reduced phosphorus oxidizing bacteria in anoxic sediments were similar to those in soils. I isolated 19 bacteria able to grow on reduced phosphorus sources, including Proteobacteria (*Pseudomonas, Acinetobacter, Variovorax,* and *Bradyrhizobium*), and two actinobacteria, suggesting a far wider phylogenetic occurrence of reduced phosphorus oxidation than previously known. To detect pathways responsible for reduced phosphorus oxidation, I characterized one gene responsible for hypophosphite oxidation, *htxA*, and one gene for phosphite oxidation, *ptxD*, in these isolates. These genes have previously only been described for a few closely related taxa. I found all isolates possessed a *ptxD* ortholog, though not all were capable of growth on phosphite. Partial sequence analysis showed *ptxD* was 100% identical to one previously characterized. Thirteen isolates possessed *htxA*, though two were not capable of growth on hypophosphite. My results indicate reduced phosphorus oxidizing bacteria and the genes required for the oxidation of hypophosphite and phosphite are abundant in the environment, and provide strong evidence for the importance of bacterial phosphorus oxidation in nature.

CHAPTER I

INTRODUCTION

A common misconception is that phosphorus (P) is inert to biological oxidation and reduction despite clear evidence that bacteria are able to oxidize and reduce P compounds (9, 21, 30, 35, 42, 44, 45, 48, 54, 55, 65-68). As a result, bacterial oxidation of reduced P compounds (Fig. 1) and the impact of this activity on P biogeochemistry have largely been overlooked. Unlike other biogeochemical cycles (e.g. nitrogen, sulfur, mercury, arsenic), in which redox reactions carried out by bacteria are central, the role of bacteria in the currently accepted P cycle is limited to the inter-conversion of inorganic phosphate (Pi, P oxidation +5) and organic Pi-containing compounds (e.g. phosphate esters, P oxidation +5) through the degradation of organic matter, in which P remains in its most oxidized state (Fig. 2).

Clearly P, particularly Pi, is biologically important, yet, Pi is often a limiting nutrient in the natural environment and microbes will often face P starvation. The most common Pi starvation processes (e.g. Pho regulons) elicit the induction of enzymes that acquire Pi from myriad P-containing compounds predominantly those with Pi in a fully oxidized state (33, 60, 70). While it is true that the majority of P compounds identified in the environment are found to contain fully oxidized P, reduced organic P compounds such as phosphonates

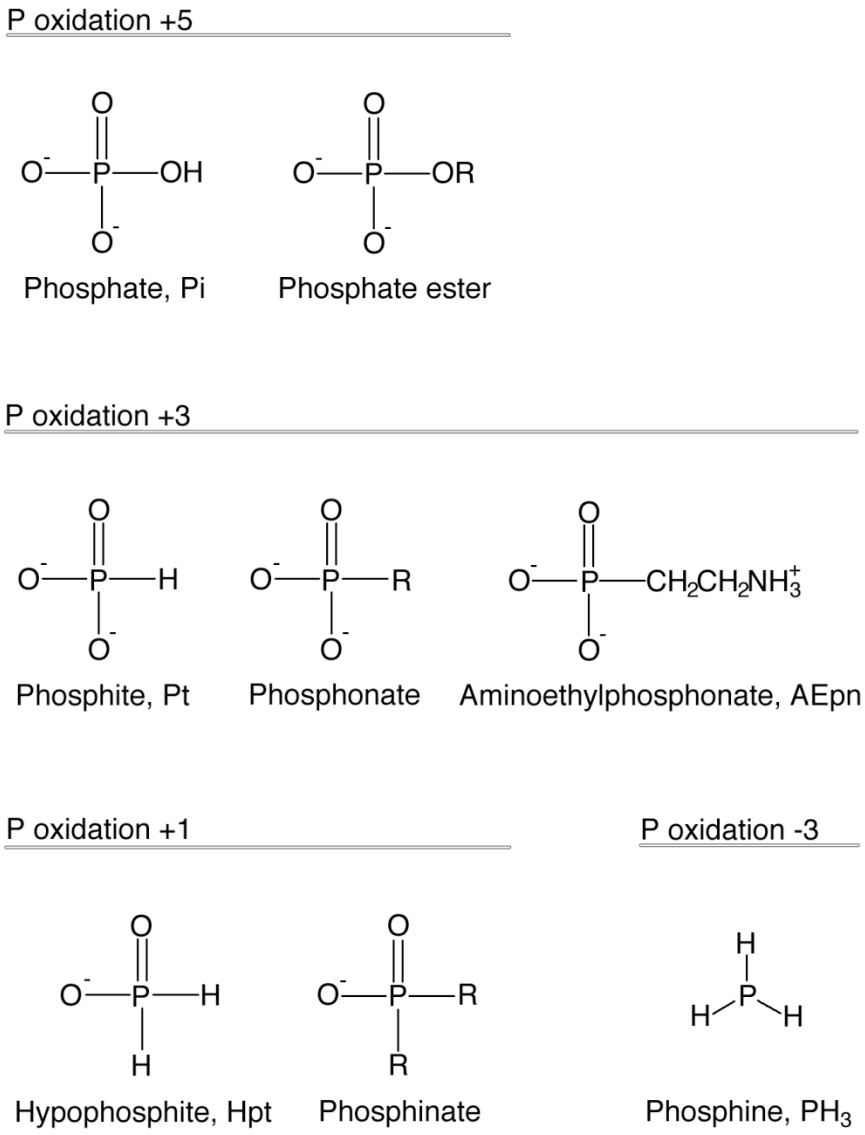

FIG. 1. Structure, oxidation states, and abbreviations of reduced P compounds used or referenced in this study.

(P valence +3), and phosphinates (P valence +1), which contain a stable carbon-phosphorus (C-P) bond in lieu of a phosphate ester bond, are also widely distributed in the environment. The existence of these reduced P compounds are due to introduction by human activities (e.g. Roundup®) and their production by soil dwelling *Streptomyces* sp., which produce a large number of diverse reduced

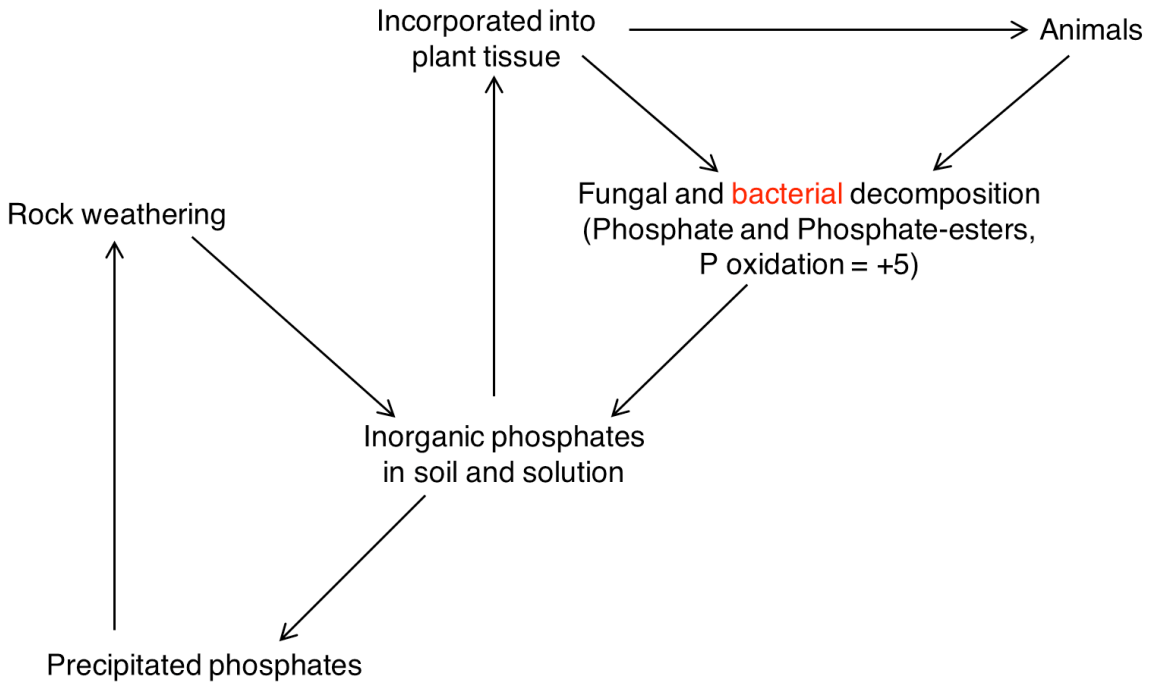

FIG. 2. Diagram showing the traditional P cycle. In a traditional P cycle only the movement of phosphate and phosphate-esters are depicted. The sole attributed role of bacteria as decomposers is shown in red.

P compounds (42).

In addition, a growing number of investigations with increasingly sensitive techniques have shown the presence of the reduced inorganic P compounds phosphite (Pt, P valence +3), hypophosphite (Hpt, P valence +1), and phosphine (PH_3, P valence -3) in numerous, particularly reducing or anaerobic environments (15, 16, 19, 24-28, 41, 42, 47, 51, 52, 65). While the natural origins of these reduced P compounds is unclear, their presence in the environment is contributed to by numerous human activities such as the use of Pt as an herbicide/fungicide and alternative P source in fertilizers, PH_3 as a fumigant/pesticide, and the use of Hpt industrially.

Bacterial Pt oxidation was first reported by Adams and Conrad (1).

Shortly thereafter, three more studies (9, 21, 30) demonstrated bacterial oxidation of Hpt and Pt. For nearly a century researchers have demonstrated various aspects of bacterial redox of P compounds (35, 42, 48, 51, 57, 58, 65). The discovery of bacteria that are able to oxidize Pt and Hpt has suggested that compounds like these are perhaps important environmental sources of P. These studies, coupled with the growing evidence that reduced P compounds are present in natural environments, suggest that reduced P oxidation (RPO) may be a ubiquitous activity among soil bacteria. However, to my knowledge, no comprehensive study investigating the abundance of reduced P oxidizing bacteria (RPOB) in the environment has yet been reported. Without this critical piece of information, the impact of bacterial redox reactions on Pi bioavailability is unknown. It follows that perhaps our understanding of P flux and transformation through ecosystems is not fully understood. Such understanding is essential for effective P conservation efforts, understanding nutrient cycling, and improving agricultural and bioremediation practices.

Molecular and Biochemical Characterizations of Reduced Phosphorus Oxidation Pathways

More recent molecular studies have shown *Desulfotignum phosphitoxidans* can use Pt as an energy source (54, 56). Meanwhile, the genetic (Fig. 3) and biochemical (Fig. 4) details of Hpt and/or Pt oxidation by *Pseudomonas stutzeri* WM88 (45, 66), *Alcaligenes faecalis* WM2072 (67, 68), and *Xanthobacter flavus* WM2814 (67) for use as a sole P source have been determined. Five mechanisms have been identified and characterized to oxidize

organic and inorganic reduced P compounds (65). The phosphonate (Phn) pathway is extremely common and has been described in diverse bacterial species where it is responsible for transportation, regulation, and cleavage of the C-P bond of phosphonates (e.g. AEpn) and phosphinates (organic reduced P compounds) but has also been shown to oxidize Pt to Pi (40, 43, 44, 61, 62, 66). Depending on the organism, 13-17 genes encode a multisubunit C-P lyase, a protein-dependent transporter, and transcriptional regulators (Fig. 3). In *Escherichia coli*, 14 genes (*phnCDEFGHIJKLMNOP,* 10.9-kb operon) encode the C-P lyase pathway. In *P. stutzeri* WM88, 13 genes (*phnCDEFGHIJKLMNP,* 10.6-kb operon) encode a single C-P lyase pathway.

A second mechanism, the *htx* operon, found in *P. stutzeri* WM88, *A. faecalis* WM2072, and *X. flavus* WM2814 is responsible for the oxidation of Hpt to Pi (Fig. 3 and Fig. 4). The *htx* operon varies between *P. stutzeri, A. faecalis*, and *X. flavus*. In *P. stutzeri* the *htx* operon consists of 16 genes, *htxABCDEFGHIJKLMNOP* (11.8-kb). Interestingly, *htxBCDEFGHIJKLMN* are homologous in function (though arranged differently) to *phnCDEFGHIJKLMNP* in *E. coli. P. stutzeri* also possesses a second *phn* operon with nearly the same arrangement as the *E. coli phn* operon (sans *phnO*). Specifically, *htxBCDE* are responsible for transporting Hpt into the cell, *htxFGHIJKLM* encodes a C-P lyase (*htxFGHIJKL* are involved in the catalysis of Hpt, *htxMN* are putative accessory proteins), and *htxOP* have no known function. Remarkably, the *htx* operon contains no known regulatory genes. *A. faecalis* has a truncated *htx-ptx* operon consisting of only *htxABCDptxDE*. The arrangement and sequence identity of

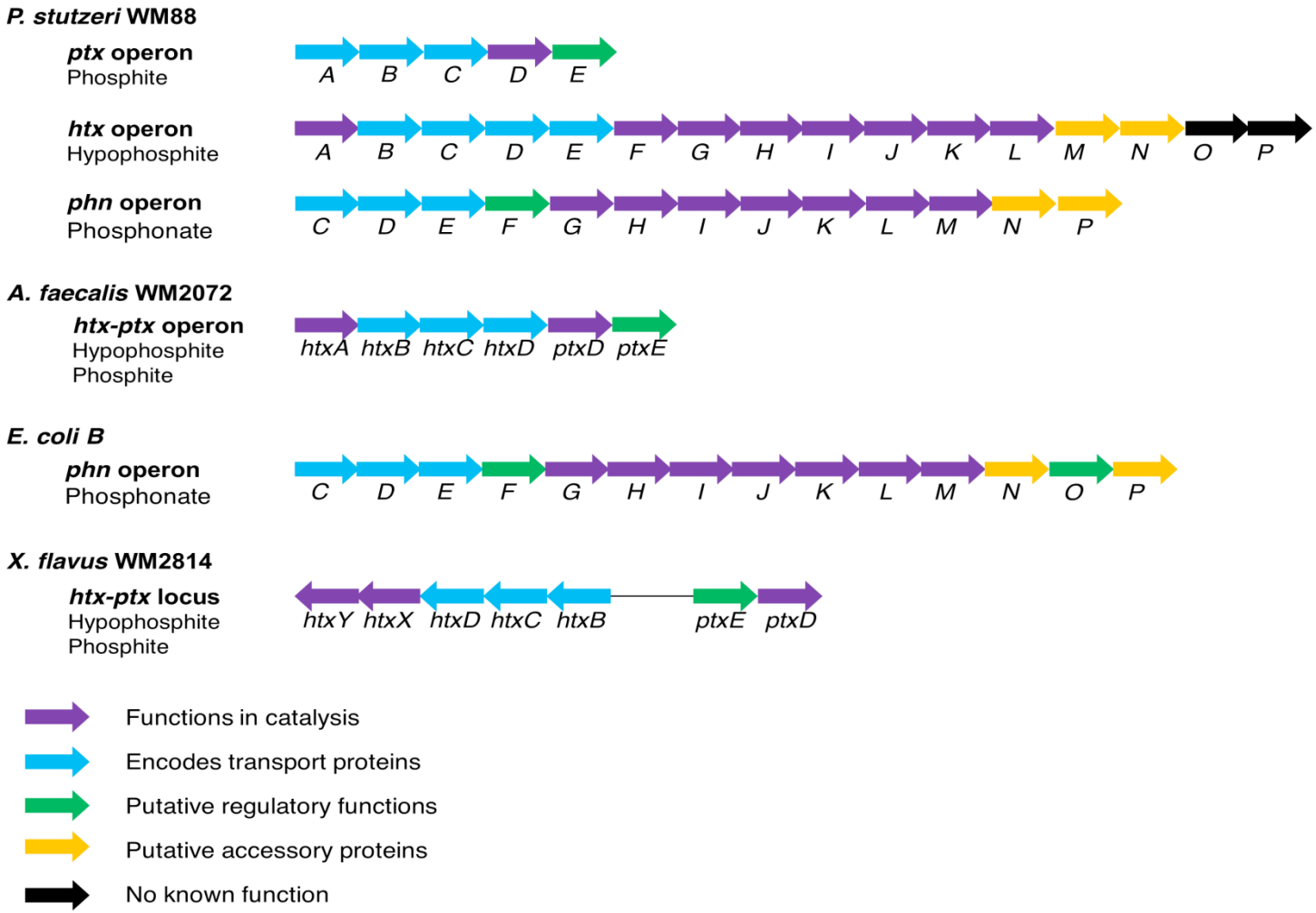

FIG. 3. Operons, loci, and substrates specific to each involved in the catalysis of reduced P compounds in *P. stutzeri* WM88, *A. faecalis* WM2072, *X. flavus* WM2814, and *E. coli B*. Key genes in this study are *htxA*, a 2-oxoglutarate dependent hypophosphite dioxygenase responsible for the oxidation of Hpt to Pt, and *ptxD*, an NAD-dependent phosphite dehydrogenase responsible for the oxidation of Pt to Pi.

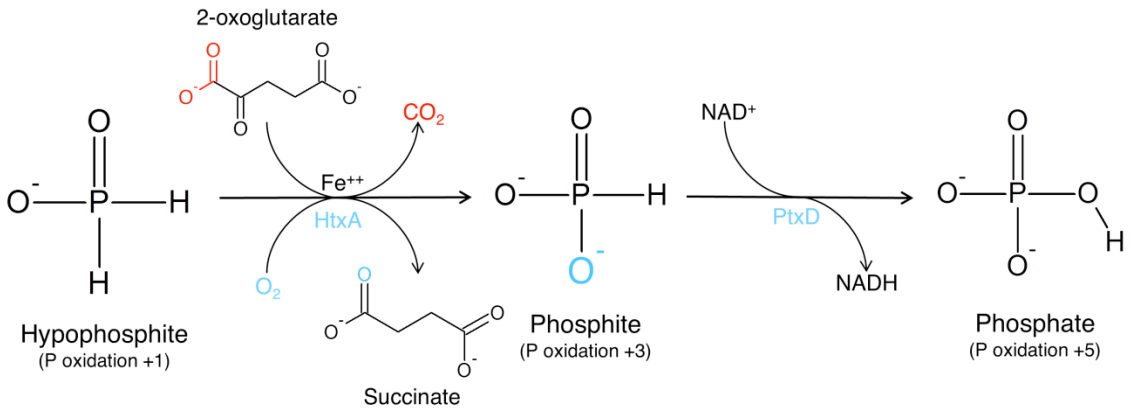

FIG. 4. Biochemical pathway for HtxA and PtxD as characterized in *P. stutzeri* WM88. *A. faecalis* WM2072 possesses the same pathway. In *X. flavus* WM2814, the Pt to Pi portion of the pathway is the same as depicted; however, it does not possess the same Hpt to Pt oxidation pathway.

htxABCD is 100% identical to *htxABCD* in *P. stutzeri*. Finally, *X. flavus* has a unique *htx* operon. The *htxBCDXY* are encoded on the reverse strand and there is no *htxA*. The *htxA* gene, a 2-oxoglutarate-dependent hypophosphite dioxygenase, oxidizes Hpt to Pt, and has been identified in *P. stutzeri* WM88 and *A. faecalis* WM2072. In both organisms, *htxA* is 100% identical at the nucleotide and amino acid level. *X. flavus* WM2814 has *htxXY*, which oxidizes Hpt to Pt. Unlike *htxA, htxXY* is an NAD-dependent hypophosphite dehydrogenase.

The final three mechanisms deal with the oxidation of Pt to Pi and include bacterial alkaline phosphatase (BAP) and *ptxD*. BAP, encoded by *phoA,* has been found in *E. coli* to oxidize Pt (71). Despite BAP enzymes from diverse organisms being tested, the ability of BAP to oxidize Pt has only been documented in *E. coli* (71).

The *ptx* operon, responsible for the oxidation of Pt to Pi, has been described in *P. stutzeri* WM88*, A. faecalis* WM2072*,* and *X. flavus* WM2814 (Fig.

3 and Fig. 4). As with the *htx* operon, the *ptx* operons are varied among these three bacteria. *P. stutzeri* has a dedicated 4.4-kb *ptx* operon; *A. faecalis* only possesses *ptxDE* directly downstream of *htxABCD,* but part of the same 5.48-kb *htx-ptx* operon; and *X. flavus* has *ptxED* that is downstream of the *htx* locus. In all three organisms, the *ptxD* gene, an NAD-dependent phosphite dehydrogenase responsible for oxidizing Pt, are each ca. 50% identical at the amino acid level. *Desulfotignum phosphitoxidans*, capable of oxidizing Pt as an energy source, has been found to have a *ptxD* ortholog, which is 39% and 40% identical at the amino acid level to the PtxD of *A. faecalis* and *P. stutzeri*, respectively.

In *P. stutzeri* WM88 and *A. faecalis* WM2072, *htx* and *ptx* are regulated by the two-component PhoBR system (64, 67, 68). The PhoBR system, induced as a response to Pi-starvation and characterized extensively in *E. coli* and other Gram-negative bacteria, prompts the expression of genes that allow alternative sources of Pi (e.g. phosphate esters) to be obtained. Pho regulon genes are repressed under Pi-rich conditions due to the saturation of PstS with Pi. The mechanism of repression is a repressor complex consisting of $PhoR^R$ (repressor PhoR), PhoU, and PstSCAB. When extracellular Pi concentrations become low, PstS is no longer saturated with Pi, and the PhoBR Pi-starvation response is elicited. PstS, part of the PstSCAB complex undergoes a conformational change, which in turn causes a change in and dissociation of $PhoR^R$ from the PstSCAB repressor complex. The dissociated $PhoR^R$ then dimerizes with another PhoR to form $PhoR^A$ (activator PhoR). The histidine of $PhoR^A$ is auto-phosphorylated, which then phosphorylates the aspartate of PhoB to form PhoB~P. The PhoB~P

then binds DNA and induces transcription of Pho regulon genes.

Clearly, Pi-starvation mechanisms are important. They are found in enteric bacteria that may face Pi-starvation less often than environmental bacteria. Understandably, it makes sense that Pi-starvation mechanisms would be especially important for "true" environmental bacteria to thrive in harsh environments such as soil, sediment, and water. With the intricate regulatory overlap between *htx*, *ptx*, and the PhoBR systems, it seems *htx* and *ptx* are potentially important and understudied Pi-starvation mechanisms for environmental bacteria. If reduced P compounds are common (or relatively common) in sediments and soils, it would only make sense that these bacteria would benefit by possessing mechanisms that would allow for the use of diverse P compounds, including Hpt and Pt. As with the abundance of reduced P oxidizers in the environment, there has been no published determination of the abundance and diversity of *ptxD* and *htxA* in the natural environment. These deficits in knowledge have led to the two questions addressed in this study.

Questions

How abundant are culturable RPOB in a variety of soils and sediments? A culture-dependent enrichment of RPOB approach was used to address this question. Specifically, I used a most probable number (MPN) method to estimate concentrations of RPOB from 12 aquatic and terrestrial environments. It is extremely important to note that the point of this study was not to characterize the individual sites chosen. Rather, I attempted to determine the

abundance of reduced P oxidizers in common environments.

How common are the previously characterized *ptxD* and *htxA* genes among bacteria in the natural environment? To address this question I used an approach to detect RPO pathways in 19 isolates cultured on reduced P sources. Specifically, PCR, hybridization, and sequencing were used to detect and characterize *ptxD* and *htxA* from environmental bacterial isolates.

CHAPTER II

MATERIALS AND METHODS

Media and Growth Conditions

All media and buffers for this study were prepared as previously described (61) unless otherwise stated. Two types of samples were used: (1) sediment, which was defined as soil covered by water (e.g. creek bed, lake bed); and (2) soil, which was defined as soil not covered by water (e.g. bank soil). Soil slurry referred to soil that had been suspended in a small aliquot of buffer to aid in pipetting or was the consistency of mud. For MPN analysis, samples were prepared as follows to remove debris and cell-free Pi: one mL of sediment or soil slurry was diluted in 9 mL 1X"M" buffer (40 mM 3-[N-morpholino]propanesulfonic acid [MOPS] based) and vortexed (61). The sample was set aside for approximately 5 minutes to allow sediment to settle. The supernatant was pre-filtered twice using Whatman filter paper, pore size 5.0 µm, lot# 8612932, into a new tube to remove additional sediment debris. Approximately 1 g of PŪRA PhosLock™ granules (ferric hydroxide to bind and remove cell-free Pi; Magnavore Company) was added to the supernatant and incubated for 1 hour at room temperature with gentle agitation. The remaining supernatant was filtered through a Nalgene® 0.2 µm pore filter by vacuum filtration. Collected cells were then resuspended from the filter into 1X"M" MOPS buffer (61). For genetic

analyses, samples were prepared as described except the supernatant was not pre-filtered and no PŪRA PhosLock™ granules were used to remove cell-free Pi. All bacteria grown on reduced P compounds were incubated at 27^{o}C for 7 days.

Most Probable Number for Reduced Phosphorus Oxidizing Bacteria

Direct plating was initially used to culture and quantify RPOB. However, it is a method that is prone to error and the resources that must be used to obtain a high number of replicates would be far greater than with other methods, such as MPN. In addition, because agar is an impure substance, trace amounts of Pi may be present and many bacteria, particularly soil bacteria, are extremely adept at scavenging trace amounts of Pi. As a result, background growth would occur on P-free agar plates, making the interpretation of Pi, Hpt, Pt, and AEpn agar plates difficult. All of these issues make the use of liquid media and MPN quite appealing. MPN is the standard method in food, water, and soil microbiology for determining the concentration of particular bacteria in a sample (20, 34, 36). MPN requires at least one observable and distinct characteristic that is unique to the target population. For this study, that characteristic was the ability to grow only in the presence of the reduced P compound Hpt, Pt, or AEpn. In the presence of Hpt, Pt, or AEpn, growth can only occur if the bacteria can oxidize the reduced P compound to Pi.

Site descriptions. Twelve samples were obtained from nine aquatic and terrestrial locations in Butte and Glenn County (Table 1 and Fig. 5). The

sample area consisted of a variety of sites including flowing and still water, large and small water bodies, and pristine to non-pristine areas. The selected sites were primarily located along Big Chico Creek, which flows from the Sierra Nevada foothills through Chico and into the Sacramento River. The watershed and sample locations within it follow a concomitantly increasing hydrological discharge and disturbance gradient due to urban and agricultural run-off. In determining the pristineness of sites, a qualitative assessment taking into account the amount of potential run-off as well as the sample type (sediment or soil) was performed. Sed1 was defined as a relatively pristine site because of upstream features. Upstream of this location is the Big Chico Creek Ecological Reserve and very little urban or agricultural run-off compared to the other sites in this study. Soil data obtained from the United States Department of Agriculture's National Conservation Service Soil Data Mart database (http://soildatamart.nrcs.usda.gov) show the 12 sites are typical (i.e. not extreme) environments (Table 2). Within the 12 sites pH values ranged from 5.1-8.4; cation exchange capacity ranged from 3.9-50 milliequivalents/100 g; salinity ranged from 0-0.5 mmhos/cm; clay ranged from 2-60%; and organic matter ranged from 0.5-6.0%.

Soil data. To characterize the 12 environments chosen and show they are typical environments, soil data was obtained from the United States Department of Agriculture's National Conservation Service Soil Data Mart database (http://soildatamart.nrcs.usda.gov). The Soil Data Mart database is the

TABLE 1. Locations of sample sites in Glenn and Butte County, California

Sample ID	Location Name/Description	Approximate Coordinates
Sed1	BCC directly south/downstream of the Big Chico Creek Ecological Reserve	39° 48' 15.43" N/ 121° 43' 43.84" W
Sed2 Soil3	Lindo Channel near 5 Mile	39° 45' 44.87" N/ 121° 47' 37.01" W
Sed3	Thermalito Afterbay	39° 28' 12.79" N/ 121° 39' 35.03" W
Sed4	Big Chico Creek on the California State University Chico Campus	39° 43' 49.98" N/ 121° 50' 42.14" W
Sed5	Horseshoe Lake	39° 46' 19" N/ 121° 46' 52" W
Sed6	Feather River between Palm and Almond Ave.	39° 24' 23.30" N/ 121° 37' 04.65" W
Sed7 Soil2	Big Chico Creek Access Boat Ramp	39° 42' 17.94" N/ 121° 56' 20.17" W
Sed8 Soil1	Irvine Finch Boat Ramp	39° 45' 00.42" N/ 121° 59' 50.14" W
Sed9	Sacramento River upstream of Big Chico Creek confluence	39° 42' 16.19" N/ 121° 56' 35.07" W

most extensive database for soil characterization available with numerous parameters determined. Five relevant chemical and physical properties were collected from the Soil Data Mart database for each site (Table 2). These properties show the chosen sites are typical (i.e. not extreme) environments. It should be noted that like many environmental characteristics, most, if not all, of the properties reported here are interrelated. For example, pH affects cation exchange capacity (CEC) and soil texture (% clay) affects CEC.

The optimal pH for bacterial activity, plant growth, and nutrient availability is approximately 6-7. Cation exchange capacity (CEC) measures the

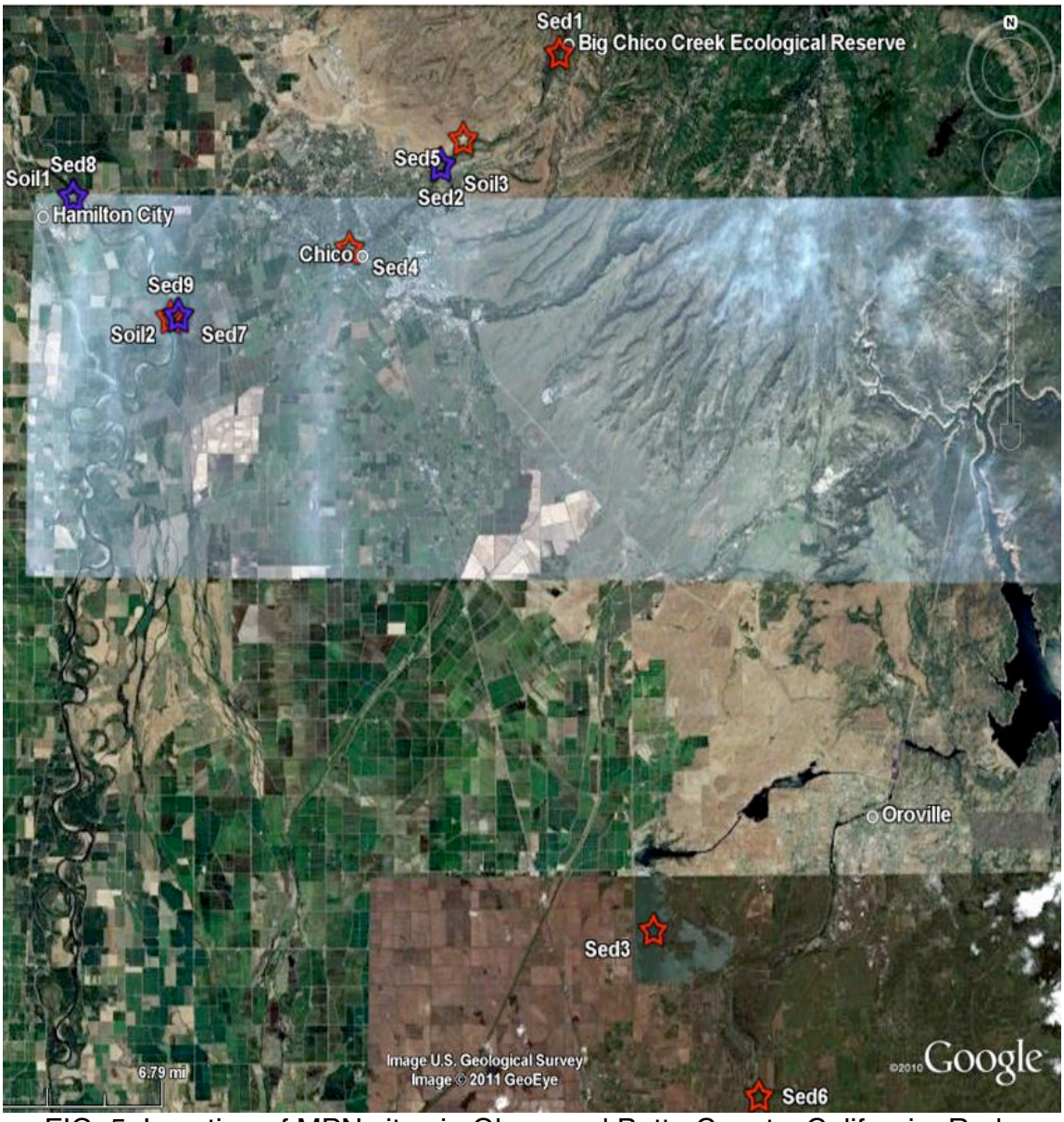

FIG. 5. Location of MPN sites in Glenn and Butte County, California. Red stars indicate sites where only sediment was collected; Blue stars indicate sites where sediment and soil were collected. Image obtained from Google Earth.

capacity of soil to retain cations at pH 7 and is reported as milliequivalents per 100 g soil (meq/100 g). Thus, CEC is a measure of the fertility of the soil, the capacity to retain nutrients, soil texture, and, ultimately, microbial communities. For agricultural soils, 10-30 meq/100 g is optimal. Salinity measures soluble salts

TABLE 2. Soil properties from the Soil Data Mart database for 12 natural environmental sites in Butte and Glenn County showing these are typical (i.e. not extreme) sites

Sample	MUSYS[a]	pH	CEC (meq/100 g)	Salinity (mmhos/cm)	% Clay	% Organic matter
Sed1	648	6.1-7.3	25-40	0	20-27	4.0-10
Sed2 /Soil3[b,c]	300	6.1-7.8	20-30	0-0.5	16-24	1.6-5.0
	991	6.1-7.3	10-20	0	2-10	1.0-4.0
Sed3	603	5.6-6.5	8.0-13	0	15-24	0.5-2.0
		5.1-6.5	6.4-11	0	12-20	0.5-2.0
		5.6-6.5	8.0-9.7	0	15-18	0.5-1.2
		5.6-6.5	6.4-12	0	12-22	0.5-1.2
Sed4	425	6.1-8.4	11-18	0-0.5	12-20	2.5-5.0
Sed5	676	6.6-7.8	40-50	0-0.5	40-59	2.0-5.0
		6.3-8.0	40-50	0-0.5	40-60	2.0-5.0
		6.6-7.8	15-30	0	18-35	2.0-6.0
		6.7-7.8	15-30	0	20-30	2.0-6.0
	991	6.1-7.3	10-20	0	2-10	1.0-4.0
Sed6	118	5.1-7.3	7.4-12	0	10-16	0.5-1.0
Sed7/Soil2	152	5.6-8.4	3.9-18	0-0.5	4-20	1.0-5.0
	158	5.6-8.4	3.9-18	0-0.5	4-20	1.0-5.0
Sed8/Soil1	ChA	6.6-7.3	5.0-15	0	10-18	0.5-2.0
Sed9	152	5.6-8.4	3.9-18	0-0.5	4-20	1.0-5.0

[a] The map unit symbol (MUSYS) is used in the Soil Data Mart database as a unique identifier for each soil type.
[b] Locations with multiple MUSYS were on the boundary of all noted MUSYS so, chemical and physical properties for each MUSYS are presented.
[c] Indicates sediment and soil samples were obtained from the same location.

in soils and is reported as millimhos per centimeter at 25^{o}C (mmhos/cm). Non-saline soils are typically defined as having salinity of 0-4 mmhos/cm. Clay is one of three components of soil texture (clay, sand, silt) and is reported as an estimate of the percentage of the total soil weight of particles with a diameter of less than 2 mm. A soil higher in clay content is more structured and thus more

conducive to microbial communities forming than soils high in silt or sand. Typically, a soil is classified as clay if it has more than 40% clay. No optimum percentage of clay for microbial communities could be found. The percent organic matter is an estimate of the percentage of the total soil weight of plant and animal residue with a diameter of less than 2 mm. Organic matter affects water capacity and infiltration, microbial and plant soil communities by providing carbon and nitrogen, and overall healthy soil. The optimum percent organic matter for agriculture is approximately 5-10%.

Validation of MPN method. To verify that the MPN method would produce consistent results, a preliminary experiment was performed using *P. stutzeri* WM88 with Luria-Bertani (LB) media. An aliquot of an overnight culture of *P. stutzeri* was suspended in 1X"M" until barely turbid. Using a 5-tube MPN dilution series with 12 dilutions (10^{-1} to 10^{-12}) per tube, the cell suspension was serially diluted 1:10 into a 96-well plate containing LB. The same cell suspension was also serially diluted 1:10 and plated onto LB agar plates with five plates per dilution. Plates were incubated overnight at 27^{o}C.

Sampling of sites. Twelve samples of sediment (soil beneath water) and adjacent bank soil prone to seasonal flooding were collected during July 2009, December 2009, and October 2010. The samples collected in July 2009 were initially used to determine the validity of the sampling method for quantifying RPOB from environmental samples. Validity was defined as consistent results within each MPN series (e.g. all Pi tubes for sample Sed1 did not vary greatly with one dilution series having five positive tubes and another having one

positive tube, rather all dilution series had four to five positive tubes).Once the validity of the sampling method was determined using the July 2009 samples, more sites were sampled in December 2009. Soil1 was initially analyzed in December 2009. However, results showed the MPN/g for Hpt, Pt, and AEpn were higher than the MPN/g for Pi. This prompted a reanalysis of Soil1 in October 2010. Soil1 was obtained in duplicate and analyzed in triplicate.

For each sample, three sediment or adjacent bank soil samples were obtained approximately 2 inches below the surface from each site from three random spots, then thoroughly mixed together to form one representative sample from each site.

Plating samples. A 5-tube MPN dilution series with six dilutions (10^{-1} to 10^{-6}) for each tube was performed for each cleaned sample (Fig. 6). Samples were serially diluted 1:10 into 96-well plates with MOPS minimal media containing 10 mM sodium pyruvate (CAS No. 113-24-6) and 1 mM of one of four P sources: Pi (Dipotassium phosphate, CAS No. 7758-11-4), Pt (Phosphorus acid, CAS No. 10294-56-1), Hpt (Sodium hypophosphite monohydrate, CAS No. 10039-56-2), or AEpn ((2-Aminoethyl)phosphonic acid, CAS No. 2041-14-7) (61). Phosphorus free (P-free) media was also inoculated for use as a negative control. The plates were incubated at 27^{o}C with gentle agitation. Growth was scored by qualitatively observing turbidity after 7 days.

Analysis. MPN and 95% confidence interval (CI) values were determined using Briones and Reichardt (5). A two-tailed *t*-test was performed for Soil1 replicates. No growth was observed in P-free media, thus no data are

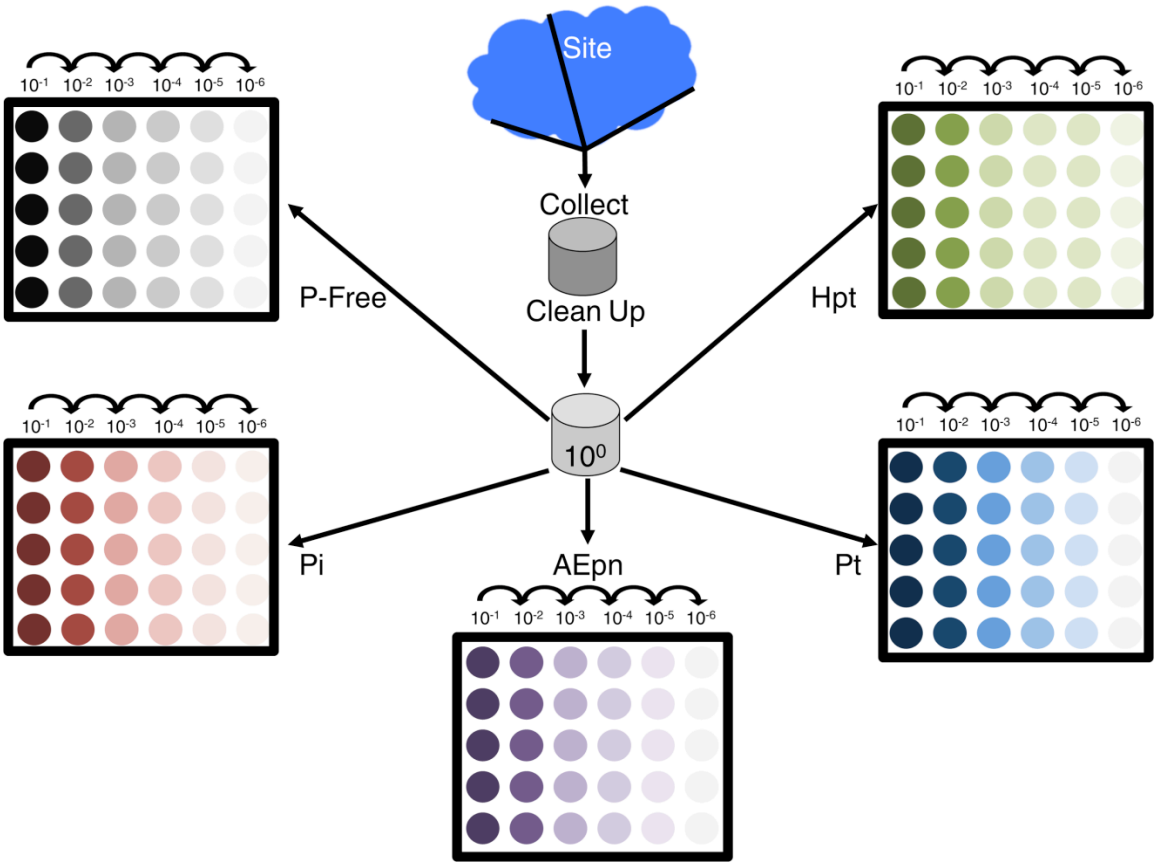

FIG. 6. Diagram of MPN experimental setup. Briefly, three samples were collected at random locations within a site (see "Sampling of sites"), cleaned (see "Media and growth conditions"), then plated in MOPS minimal media containing no P, Pi, Hpt, Pt, or AEpn (see "Plating samples"). Each dilution (columns) had 5 replicates (rows).

presented for P-free replicates. Charts were created using SigmaPlot (version 12.0; Systat Software Inc., San Jose, CA).

Genetic Analyses for *htxA* and *ptxD*

Bacterial strains used and obtained in this study. The bacteria used in this study were isolated from sediment obtained from Klamath Lake, Oregon. Cells resuspended from cleaned sediment were directly plated in triplicate on agar plates containing Pi, Hpt, Pt, or AEpn. P-free agar plates were

also inoculated. Bacteria were restreaked until pure colonies were obtained. Each isolate was identified to the species level by sequencing 1495 bases of the 16S rRNA gene using degenerate primers (27F: GTTTGATCMTGGCTCAG; 1522R: AAGGAGGTGATCCANCCRCA). Identifications were based on forward and reverse strand sequence (See "Sequence analysis of 16S rRNA, *htxA*, and *ptxD* PCR products" in Methods). Control strains (*P. stutzeri* WM88 [wt, *htxA*$^{+}$*ptxD*$^{+}$], *P. stutzeri* WM3617 [deletion mutant, Δ*htx* Δ*ptx* Δ*phn*] *A. faecalis* WM2072 [wt], and *X. flavus* WM2814 [wt]) were obtained from Dr. William W. Metcalf.

Determination of each isolates substrate range. The reduced P phenotypes of pure cultures were determined by inoculating each isolate into liquid MOPS media containing Pi, Hpt, Pt, or AEpn. P-free media tubes were also inoculated. No turbidity was observed in the P-free media. Growth in the reduced P media was scored after 7 days and compared to growth in Pi media. Growth was scored by qualitatively observing turbidity in each media type.

PCR and hybridization for *htxA* and *ptxD.* To determine the presence of *htxA*, *A. faecalis ptxD*, *P. stutzeri ptxD*, and *X. flavus ptxD*, PCR was performed on each isolate using primers specific for each gene (Table 3). The *X. flavus ptxD* is extremely GC-rich (72.8%) making amplification difficult. A NaOH pretreatment was initially attempted (2). This worked well for amplifying *ptxD* from *X. flavus* but proved tedious, difficult, and unreliable for the isolates. Adding DMSO to a final concentration of 5% (v/v) to the PCR reactions worked equally well for amplifying *ptxD* from *X. flavus*. It also gave products from many of the

TABLE 3. Primers and expected product size for amplified *htxA* and *ptxD* products

Primer	Primer Sequence (5'-3')	Expected Product Size (nt)
htxA SpeI	AAGCTTACTAGTTGATCGAATCAGCATGCC	936
htxA XbaI	GTTTCTAGATCAGTAGTACTTTTGAGTCAAAGC	
P. stutzeri ptxD	CCGAGTACACGATGAGATCC	953
	GGTTCGCAGCGTTGATTGGG	
A. faecalis ptxD	CGTAACAACGCATCGAATCC	934
	AACGCTTCGAGGATACTGAGG	
X. flavus ptxD	CCCGCAAGACCATCGTCACC	962
	GGTCCTGCTGCAATCCGTCC	

isolates.

A. faecalis WM2072 *ptxD*, *P. stutzeri* WM88 *ptxD*, *htxA*, 16S rRNA were amplified using a two cycle PCR program (annealing temperature of 55^{o}C for 30 cycles, then 50^{o}C for 10 cycles). This program was used for the initial amplification of *X. flavus* WM2814 *ptxD*, but produced multiple bands in most isolates. A one cycle PCR program (annealing temperature of 57^{o}C for 30 cycles) was then attempted. This cleaned up a couple isolates with multiple bands. Further troubleshooting is required for *X. flavus ptxD* PCR.

DNA hybridization was also performed for *htxA*, *P. stutzeri ptxD*, and *A. faecalis ptxD* using the DIG-High Prime DNA Labeling and Detection Starter Kit I per manufacturer's instructions (Cat. No. 11745832910, Roche Diagnostics, Indianapolis, IN). Probes were purified and labeled *htxA, P. stutzeri* WM88 and *A. faecalis* WM2072 *ptxD* PCR products. The labeling reaction was incubated overnight at 37^{o}C to increase DIG-labeled probe yield. Whole chromosomal DNA from controls and isolates were dotted and heat fixed (80^{o}C for 1 h) onto a nylon

membrane. Optimum hybridization temperatures (T_{opt}) were found to be 50°C for *htxA* and *A. faecalis ptxD*, 55°C for *P. stutzeri ptxD*, and were calculated using the following equations: $T_m = 49.82 + 0.41$ (%GC) – (600/l) where l = length of hybrid in bp, and $T_{opt} = T_m – 20$°C. The hybridization reactions were incubated at the appropriate temperature (see above) overnight with gentle agitation. Membranes were then washed under constant agitation first at low stringency (5 min in 2X SSC with 0.1% SDS at room temperature twice) then at high stringency (15 min in 0.5X SSC with 0.1% SDS at 66°C twice). Detection was performed using the color development substrates nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP).

Sequence analysis of 16S rRNA, *htxA,* and *ptxD* PCR products. PCR products were cleaned using the QIAquick PCR Purification Kit per manufacturer's instructions (Cat. No. 28104, Qiagen Inc., Valencia, CA) and run on a 1% TAE agarose gel stained with ethidium bromide to quantify the amount of purified PCR product, which was then sent to the San Diego State CSUPERB Microchemical Core Facility to be sequenced. *A. faecalis ptxD* products from three isolates were sequenced twice to ensure sequence validity. Chromatograms were visually inspected using FinchTV 1.4.0 (Geospiza, Inc.; Seattle, WA; http://www.geospiza.com) then aligned using ClustalW (29, 38).

A region of ca. 500 bp acquired from sequencing and aligning forward and reverse strands was obtained for all 16S rRNA sequences. This region was then searched in the Ribosomal Database to determine the identity of isolates (10, 11). A region of 838 and ca. 815 bp obtained from sequencing and aligning

forward and reverse strands was obtained for all *ptxD* and *htxA* sequences, respectively. Sequences were then aligned and percent identifies were found using ClustalW.

Database mining for DNA similar to *htxA* and *ptxD.* Three methods were used to find sequences similar to *htxA* and the three *ptxD*s in the NCBI database (http://www.ncbi.nlm.nih.gov/): (1) Searching three NCBI BLAST nucleotide databases (Nucleotide collection [nr/nt], Whole-genome shotgun reads [wgs], and Environmental samples [env_nt] databases at http://www.ncbi.nlm.nih.gov/BLAST); (2) Searching *htxA* or *ptxD* in the NCBI Nucleotide database; and (3) Searching the terms “phosphite dehydrogenase,” “phosphite,” “hypophosphite,” or “hypophosphite dehydrogenase” and reviewing each result for annotations specific to *htxA* or *ptxD*.

P. stutzeri WM88 and *A. faecalis* WM2072 *htxA* DNA sequences, as well as *ptxD* DNA sequences from *P. stutzeri* WM88, *A. faecalis* WM2072, and *X. flavus* WM2814 were searched in the NCBI BLAST database. Once BLAST results were obtained, a minimum query coverage of 80% was required for sequence matches to be included in alignments with control and sequenced (this study) *ptxD* and *htxA* products.

CHAPTER III

RESULTS

Most Probable Number for Reduced Phosphorus Oxidizing Bacteria

Validation of MPN method. MPN and viable cell counts were calculated and were within the same order of magnitude. This experiment showed I could obtain results using a 5-tube MPN dilution series consistent with a plate dilution method.

Validation of sampling method for estimating reduced P oxidizers in the environment. Two independent experiments were performed for Soil1 samples to determine the reliability of the sampling method for estimating RPOB from environmental samples (Fig. 7). A two-tailed *t*-test comparing the MPN/g soil for the same media types confirmed the samples were not significantly different ($p > 0.05$). The sampling method is a reliable method for estimating concentrations of reduced P oxidizers.

Reduced P oxidizing bacteria are present in substantial concentrations in the natural environment. The previous research (see "Introduction") strongly suggests RPOB are present in many anaerobic soils; however, no known comprehensive study has been performed to show the presence and concentration of RPOB in the natural environment. It is assumed that all culturable bacteria under the experimental conditions used are

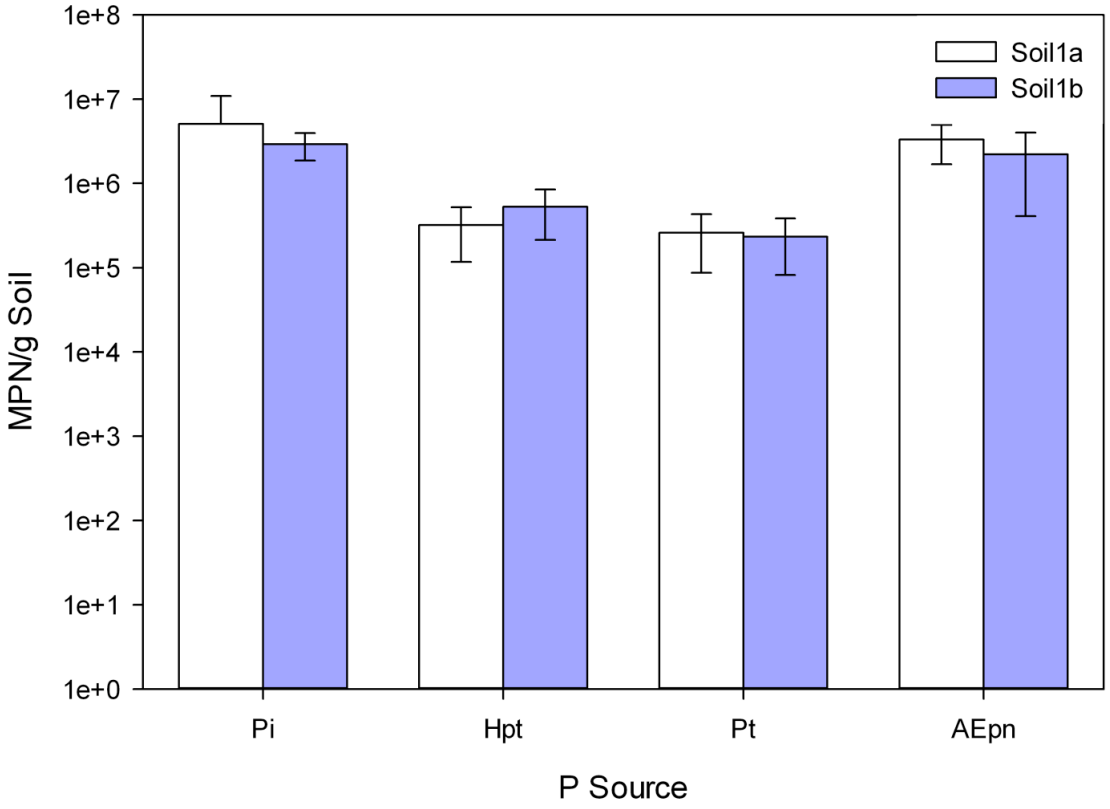

FIG. 7. MPN/g soil for sample Soil1. Two independent experiments for soil samples collected from the Soil1 site (Soil1a and Soil1b) were performed. P-free samples are not shown because no growth was observed. Each bar represents the average of three separate MPN trials. Error bars represent ± one standard deviation.

represented by concentrations observed with Pi as the P source. Since Pi is the predominate form of P in the environment, the assumption has been that if RPOB are present, they will be in extremely small concentrations relative to concentrations with Pi. However, the average concentrations of bacteria with Hpt or Pt as the sole P source are present in relatively large concentrations (Fig. 8).

Reduced P oxidizing bacteria are present regardless of the season. The collection of samples in summer and winter was simply an artifact of when samples could be collected. That is, I did not seek to determine temporal

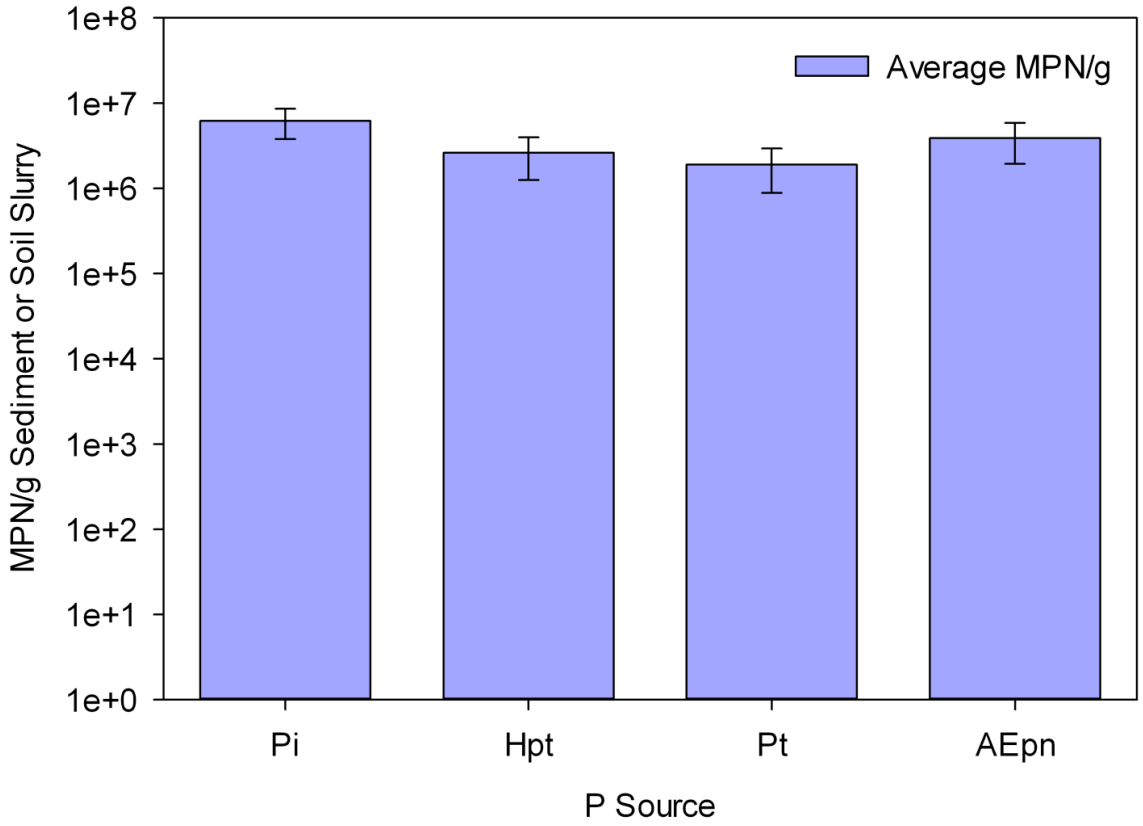

FIG. 8. The average MPN/g for 12 aquatic and terrestrial sites shows the number of bacteria capable of oxidizing Hpt, Pt, or AEpn are comparable to the MPN/g with Pi as the sole P source. Error bars represent standard error of the mean.

differences. However, I did see that regardless of the season (winter or summer), reduced P oxidizers are present (Fig. 9). Indeed, the overall pattern of concentrations with reduced P compounds, relative to Pi, is consistent in summer and winter. That is, the concentrations of Hpt and Pt oxidizers are generally lower than concentrations with Pi and AEpn concentrations are approximately equal to Pi concentrations. Since temperatures are colder in winter and rainfall increases, I would expect viable bacterial populations to decrease in winter due to increased rainfall, which coupled with colder temperatures could result in frequent freezing.

This frequent freezing would kill some of the soil populations or result in sporulation or other survival mechanisms being initiated. While culturing on rich media would result in sporulating bacteria growing, this may not be the case when cultured on minimal media as was used in this study. As a result, I expected an overall decrease in MPN/g values from summer to winter samples (Fig. 9). One reason to expect a decrease would be due to colder temperatures during winter.

Interestingly, in summer samples, when comparing only the MPN/g values and not the 95% confidence intervals, the concentrations of Hpt oxidizers are equal to or higher than concentrations of Pt oxidizers. In winter samples, again comparing only the MPN/g values, the concentrations of Pt oxidizers are equal to or higher than the concentrations of Hpt oxidizers. Still, within most samples, the 95% confidence intervals overlap indicating there are no significant differences. There were significant differences found in concentrations for three sites: Sed3, between Pt and AEpn; Sed6, between Pi and Hpt; and Soil1, between Pi and Pt. Further studies using soils or sediments from the same site collected and analyzed during summer and winter are required to determine whether the relative concentrations of Hpt and Pt oxidizers to Pi utilizers do differ between summer and winter and may provide additional, though indirect information regarding the availability of Hpt and Pt.

Microbial reduced P oxidization is not a new phenomenon. With the increasing use of commercial and agricultural products containing reduced P

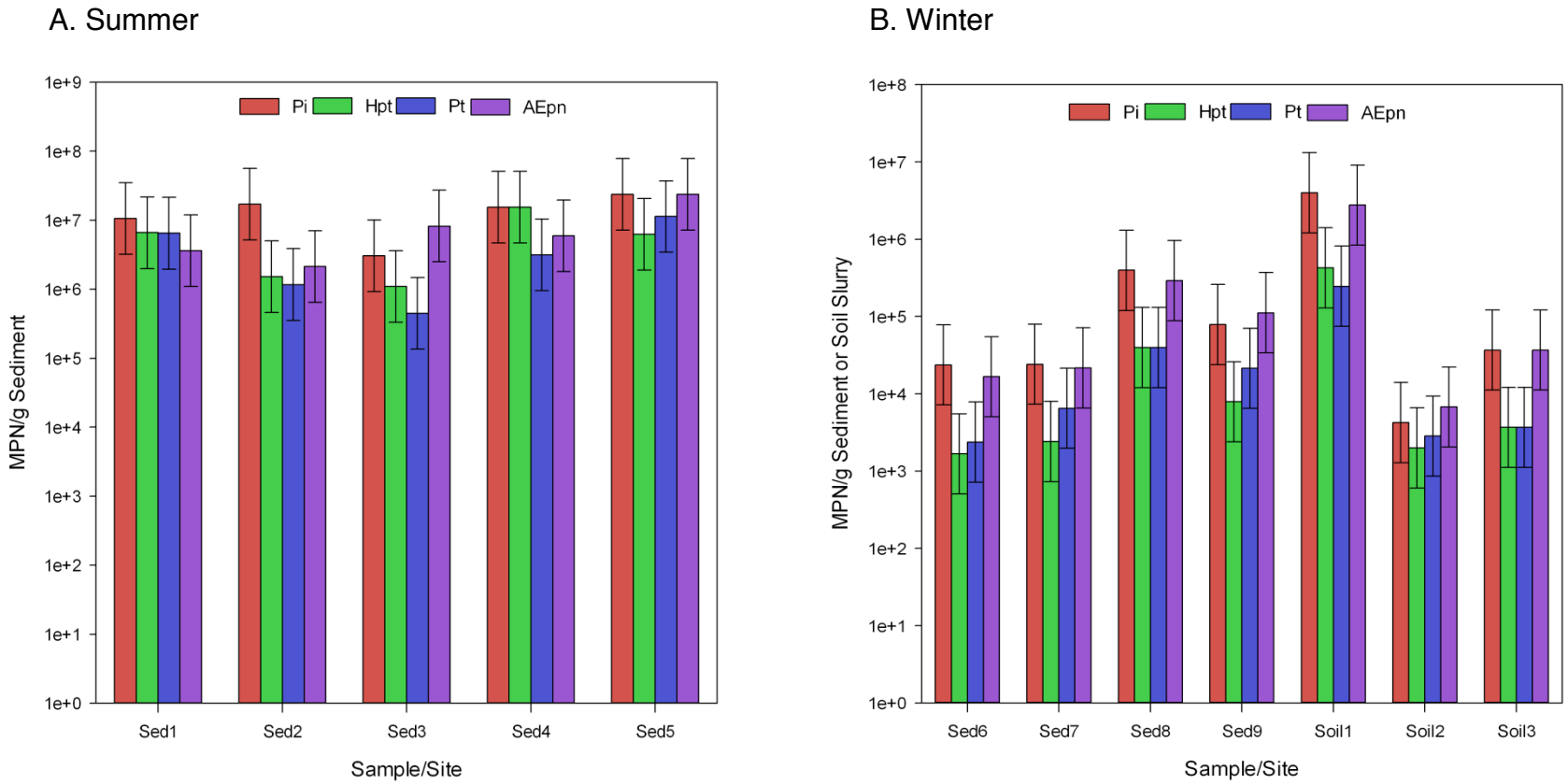

FIG. 9. MPN/g sediment or soil slurry for 12 samples collected during summer (A) or winter (B). These graphs show within each site the estimates for Hpt and Pt oxidizers are below but within the 95% CI relative to Pi. Error bars represents upper and lower 95% CI boundaries.

compounds, it could be postulated that RPO is a relatively new phenomenon. To examine this I investigated a number of sites within or downstream of numerous urban and agricultural areas, including the Chico State University Farm known to use PH_3 and Roundup® as fumigants and pesticides. If RPO were a relatively new phenomenon, driven by the introduction of these compounds through the use of products containing reduced P compounds, I would expect to see lower concentrations in areas where these products are not used. When the MPN/g from a relatively pristine site (Sed1) is compared to the MPN/g from less pristine sites, similar concentrations of RPOB, relative to Pi, are observed (Fig. 10).

Percentages normalize the data. Since samples were collected in different seasons, it can be difficult to determine distinct patterns in the data. The percentage of total culturable bacteria (represented by Pi) capable of growth on the reduced P compounds was determined (Fig. 11). The percentage observed on Hpt ranged from 7-100%, on Pt from 10-67%, and on AEpn from 34-270%. The percentages on AEpn are above 100% for Sed3, Sed9, and Soil2. This was not surprising since, as stated previously, phosphonates are extremely common in the environment and numerous bacteria are capable of using phosphonates as a sole P source. In addition, the 95% confidence intervals for Pi and AEpn MPN/g overlap (Fig. 9 and Fig. 10), indicating no significant difference between Pi and AEpn.

No differences exist between sediment and soil concentrations. For paired sediment and soil samples the concentrations of RPOB were

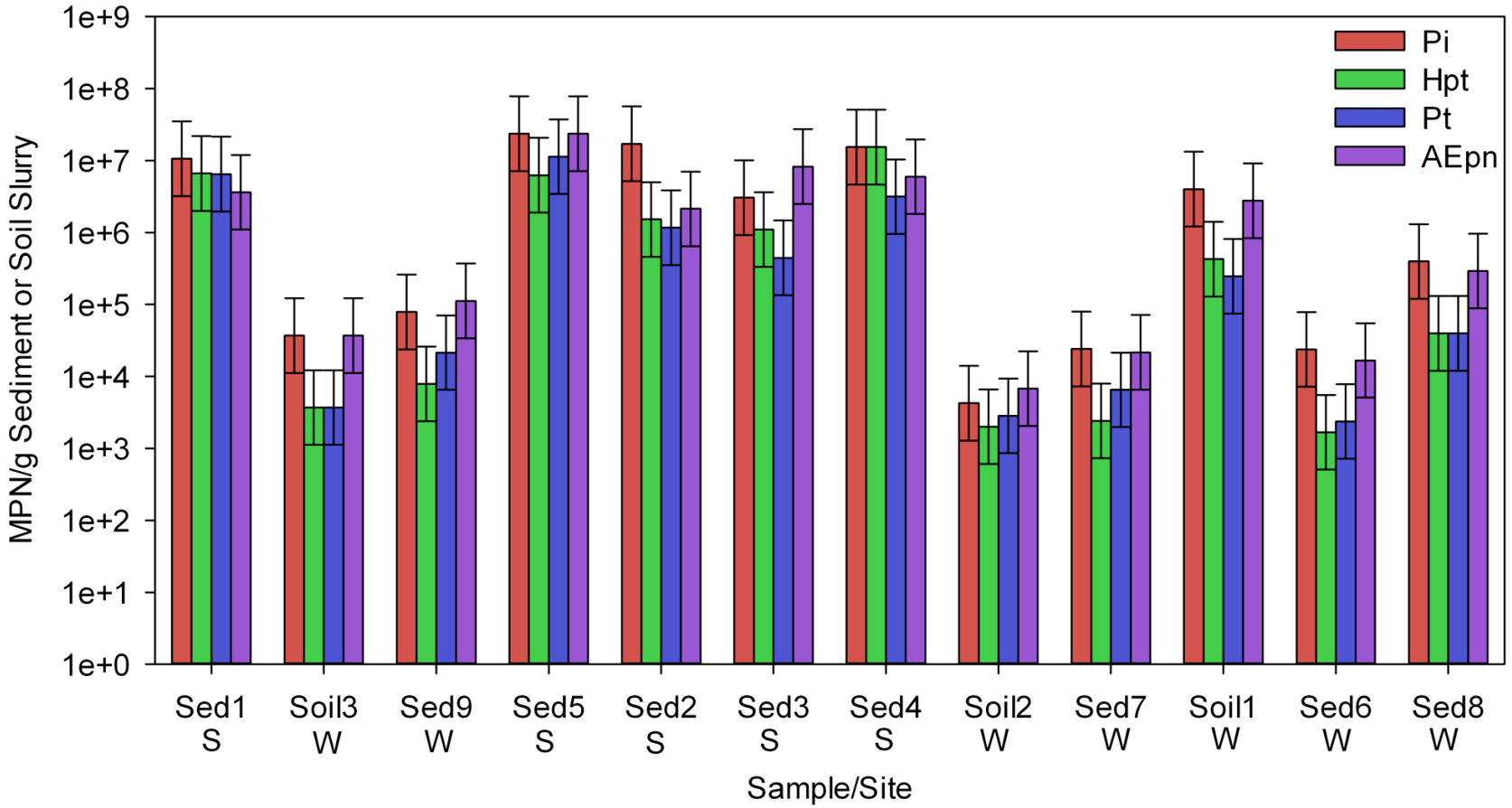

FIG. 10. MPN/g for reduced P oxidizing bacteria in each of the 12 aquatic (Sed) and terrestrial (Soil) sites arranged by most pristine (Sed1, mountain stream) to least pristine (Sed8, extensive urban and agricultural run-off). Sites were ranked based on the proximity to the pristine site (Sed1) and, consequently, the amount of potential urban and/or agricultural run-off likely to occur upstream from a particular site. Summer (S) and winter (W) sites are indicated. Error bars represent upper and lower 95% CI boundaries.

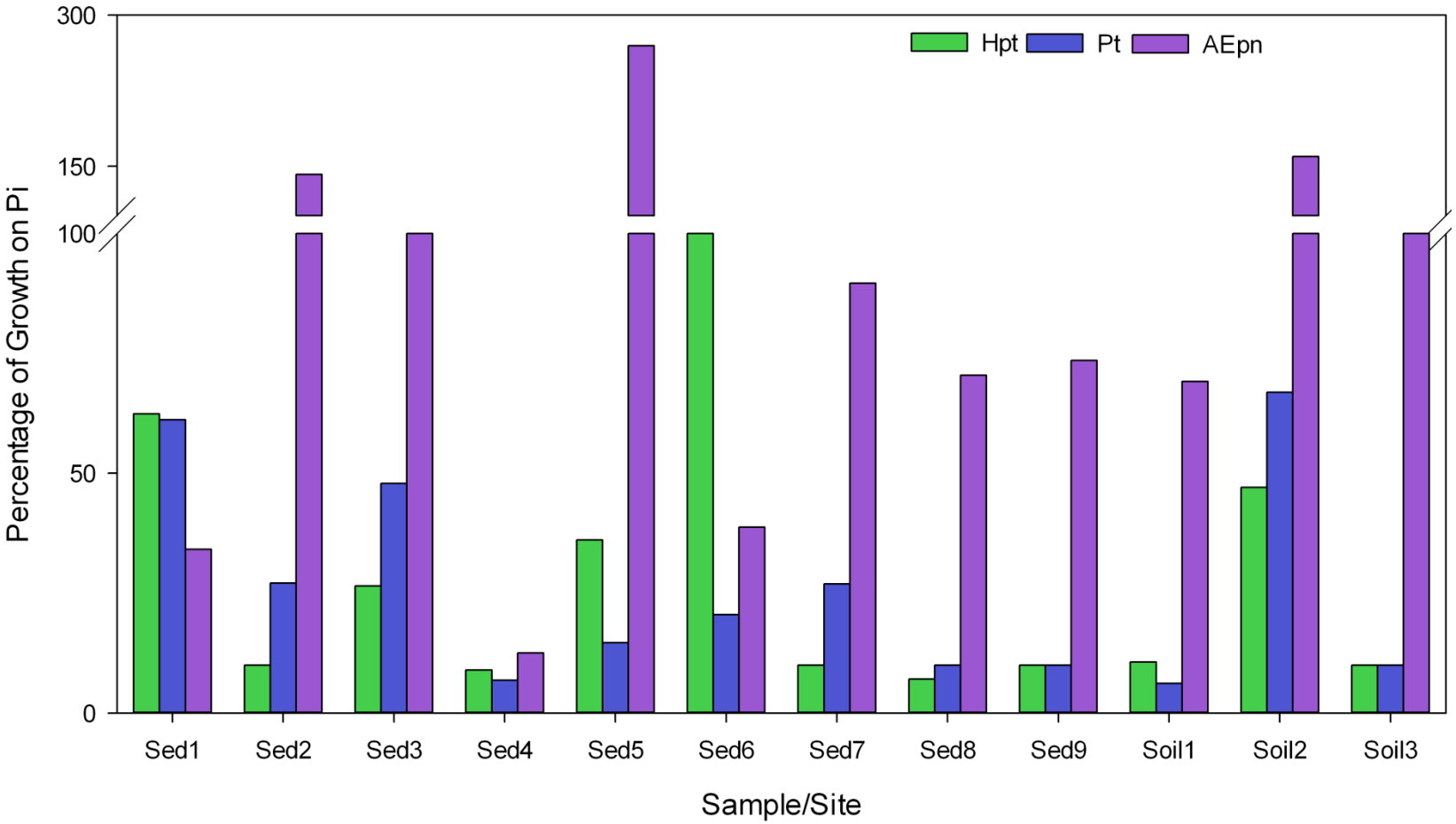

FIG. 11. Ratio of MPN capable of growth on Hpt, Pt, and AEpn expressed as a percentage of Pi (defined as 100%). For most of the sites the percentage of total culturable concentration capable of growth on Hpt or Pt is under 25%. However, for four sites (Sed1, Sed3, Sed6, Soil2) the percentage capable of growth on Hpt and/or Pt is 50% or greater. As expected, the percentage capable of growth on AEpn was generally equal to or greater than concentrations on Pi.

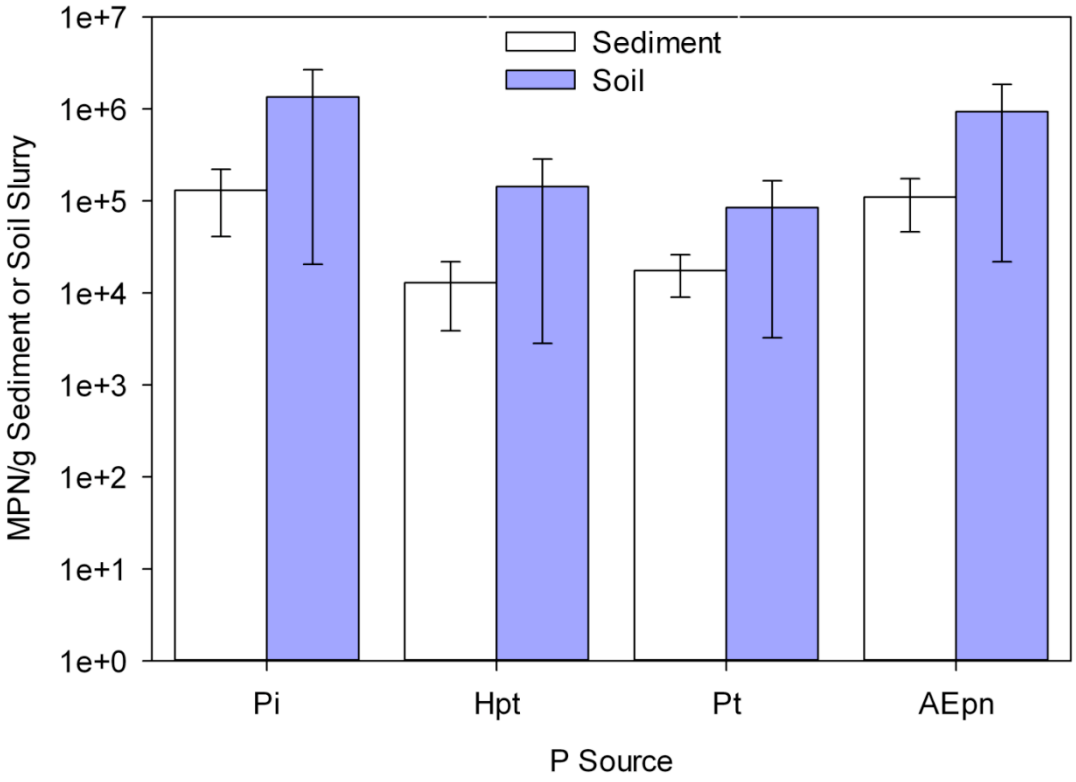

FIG. 12. Average MPN/g sediment or soil slurry for paired sites. A two-tailed *t*-test showed no significant difference among reduced P compounds for soil and sediment samples. Relative to Pi, the concentrations of RPOB are high in both sediment and soil. Error bars represent standard error of the mean.

compared (Fig. 12). Although soil samples had a slightly higher MPN/g compared to sediment samples, no significant difference was found using a two-tailed *t*-test, $p > 0.05$. It is important to note that the culture methods used allow only obligate and facultative aerobes to be accounted for even though anaerobic RPOB may also be present. Still, these data show high concentrations of RPOB are found in low and high oxygen environments.

Genetic Analyses for *htxA* and *ptxD*

Identification of cultured reduced P oxidizing bacteria. Nineteen bacteria, isolated from Klamath Lake, capable of growth using Hpt, Pt, and/or

AEpn as the sole P source were identified from 16S rRNA gene sequencing (Fig. 13 and Table 4). Most of the isolated bacteria were Pseudomonads (*Pseudomonas* sp.*, P. mendocina, P. fluorescens, P. reinekei, P. putida, P. aeruginosa, P. stutzeri*). There were also other Proteobacteria (*Bradyrhizobium elkanii*, *Variovorax paradoxus*, and *Acinetobacter calcoaceticus/baumannii* complex (Acb complex)) and two Gram-positive bacteria (*Streptomyces* sp. and *Micrococcus luteus*). Unfortunately, a species level identification was not possible for *Streptomyces* and those identified as *Pseudomonas* sp. Adequate sequence was obtained (ca. 500 nt), however, matches from searching these sequences in the Ribosomal Database Project yielded no single species identification, only genera level identifications.

The 16S identification of Gram-positive bacteria did raise questions about the validity of those two identifications, especially for *M. luteus* since the use of minimal media is not typically conducive to culturing Gram-positive bacteria (7). *Streptomyces* is an extremely common soil bacterium and generally considered a resilient Gram-positive bacterium (e.g. spore-forming, diverse physiology/metabolism). *M. luteus* is commonly classified as a skin bacterium but is easily found in soils and water. Colony morphologies for *Streptomyces* and *M. luteus* matched published descriptions (3, 49). A Gram stain showed *Streptomyces* was Gram-variable and *M. luteus* was clearly Gram-positive. Cell shape and arrangement was consistent with those described for *M. luteus* (coccus, tetrad arrangement) (3).

Determination of reduced P substrate range. The reduced P

TABLE 4. Substrate ranges, hybridization, and PCR for *ptxD* and *htxA* of 19 isolates. α, β, and γ indicate Proteobacteria class and G+ indicates Gram-positive.

Isolate	*ptxD*				*htxA*		Substrate Range[a]		
	P. stutzeri		*A. faecalis*	*X. flavus*					
	PCR[b]	Hyb	PCR	PCR[c]	PCR	Hyb	Hpt	Pt	AEpn
Acinetobacter Acb BS14 (γ)	–	–	+	+	–	–	+	+	+
Streptomyces sp. BS20 (G+)	–	–	+	+	+	–	+/–	+	+
Bradyrhizobium elkanii BS9 (α)	–	–	+	MB[d]	–	–	+	+	+
Pseudomonas aeruginosa BS16 (γ)	–	–	+	MB	–	–	–	+/–	+
Pseudomonas reinekei BS4 (γ)	–	–	+	MB	–	–	–	+/–	+/–
Acinetobacter Acb BS13 (γ)	+	–	+	+	+	–	+	+	+
Variovorax paradoxus BS2 (β)	+	–	+	+	+	–	+	+	+
Micrococcus luteus BS12 (G+)	+	–	+	+	+	–	+	–	–
Variovorax paradoxus BS8 (β)	+	–	+	+	+	–	–	+	+
Variovorax paradoxus BS6 (β)	+	–	+	MB/+[e]	+	–	+/–	+/–	+
Variovorax paradoxus BS5 (β)	+	–	+	MB/+	+	–	–	+/–	+
Pseudomonas putida BS15 (γ)	+	–	+	MB	+	–	+	+	+
Pseudomonas sp. BS18 (γ)	+	–	+	MB	+	–	+	+	+/–
Pseudomonas sp. BS21(γ)	+	–	+	MB	+	–	–	+/–	+
Pseudomonas stutzeri BS3 (γ)	–	+	–	MB	–	+	+	+/–	+/–
Pseudomonas putida BS17 (γ)	–	+	+	MB	+	–	–	–	+
Pseudomonas sp. BS19 (γ)	–	+	–	MB	–	–	+	+	+/–
Pseudomonas fluorescens BS1 (γ)	+	+	+	MB	+	+	+	+	+
Pseudomonas mendocina BS7 (γ)	+	+	+	MB	+	+	+	+	+

[a] A (+) indicates turbidity was ca. equal to turbidity with Pi. A (+/–) indicates turbidity was less than Pi. A (–) indicates no turbidity.
[b] A (+) indicates a product was obtained with primers designed from the corresponding control strain
[c] Results for *X. flavus* PCR are a combination of two separate PCR cycles.
[d] Indicates multiple bands present.
[e] Indicates multiple bands present but the strongest product was ca. 950 bp.

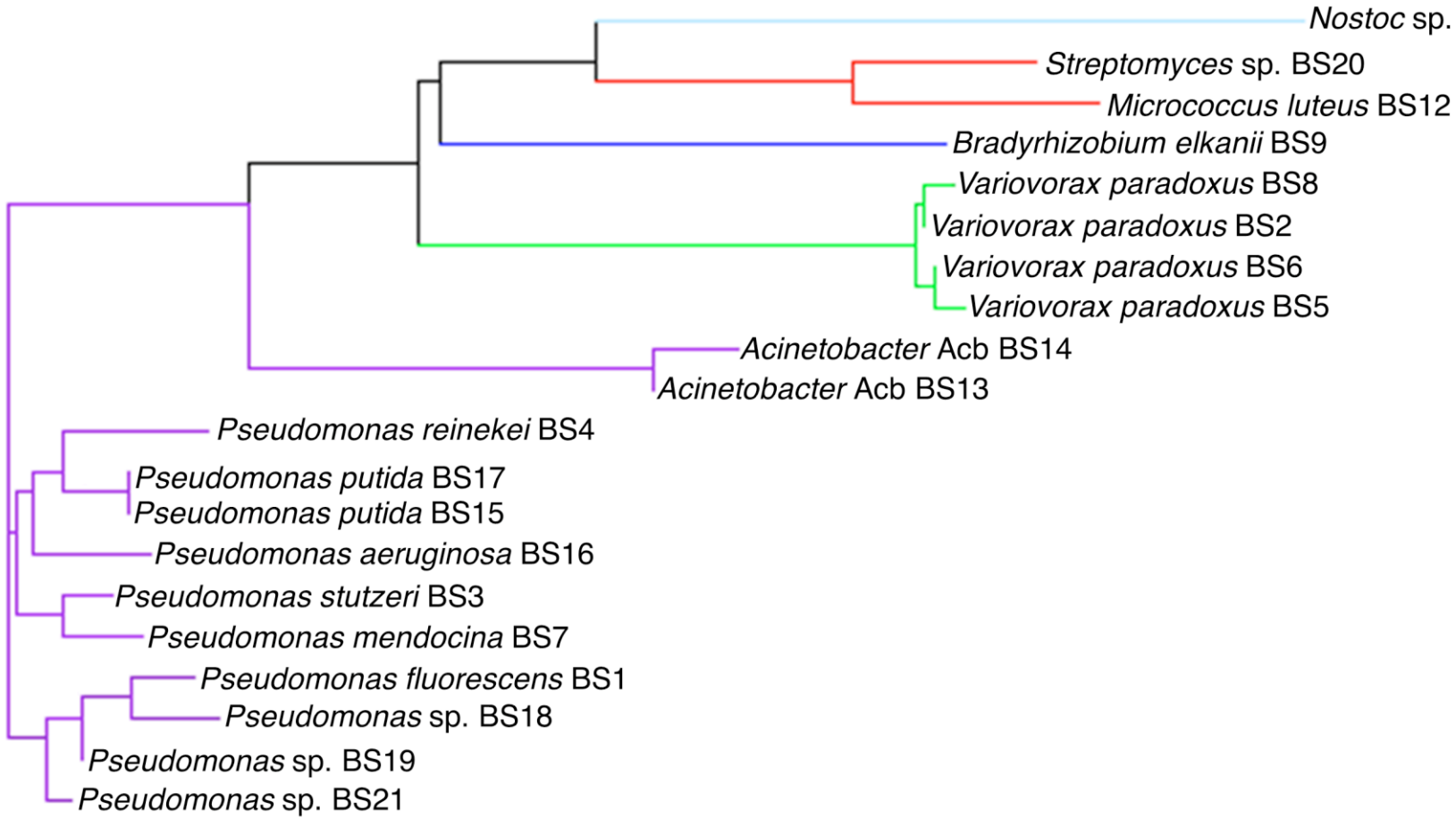

FIG. 13. 16S rRNA identifications of 19 reduced P oxidizing bacteria. Most are Pseudomonads, various Proteobacteria, and two Gram-positive bacteria. Identifications are based on ca. 500 bp (double strand [forward and reverse] sequence for species, single strand [forward or reverse] sequence for genus) of the 16S rRNA gene. The light blue line shows the outgroup (*Nostoc* sp.); red lines show Gram-positive bacteria; blue line shows α-Proteobacteria; green lines show β-Proteobacteria; purple lines show γ-Proteobacteria. Phylogenetic tree created using ClustalW. See Table A1 in Appendix A for a matrix comparison of sequence homologies.

substrate range of each isolate was determined to further characterize the isolated bacteria and elucidate potential RPO pathways (Table 4). Twelve were capable of strong (+) or moderate (+/ –) growth on Hpt, Pt, and AEpn. Five were capable of strong or moderate growth on Pt and AEpn. *Pseudomonas putida* BS17 was only capable of growth on AEpn. *Micrococcus luteus* BS12 was capable of growth only on Hpt (not Pt or AEpn).

Hybridization for *htxA.* Hybridization was performed for *htxA* for all isolates (Table 4, Fig. 14, and Fig. 15). Only *P. fluorescens* BS1 and *P. mendocina* BS7 gave a positive result with hybridization and PCR. *B. elkanii* BS9, *P. reinekei* BS4, *P. aeruginosa* BS16, *Acinetobacter* Acb BS14, and *Pseudomonas* sp. BS19 were negative with hybridization and PCR. *P. stutzeri* BS3 was positive with hybridization but negative with PCR. The remaining 11 isolates were positive with PCR and negative with hybridization.

Hybridization for *ptxD.* Hybridization was performed for *P. stutzeri* WM88 *ptxD* (Table 4). Hybridization was also performed for *A. faecalis* WM2072 *ptxD*, however, all isolates were negative. Due to the high GC content of the *X. flavus* WM2814 *ptxD*, hybridization was not performed. Only *P. fluorescens* BS1 and *P. mendocina* BS7 were positive with both PCR and hybridization for *P. stutzeri* WM88 *ptxD*. *B. elkanii* BS9, *P. reinekei* BS4, *Acinetobacter* Acb BS14, *P. aeruginosa* BS16, and *Streptomyces* sp. BS20 were negative for both PCR and hybridization. *P. stutzeri* BS3, *P. putida* BS17, and *Pseudomonas* sp. BS19 were negative with PCR but positive with hybridization. The remaining nine were positive with PCR and negative with hybridization.

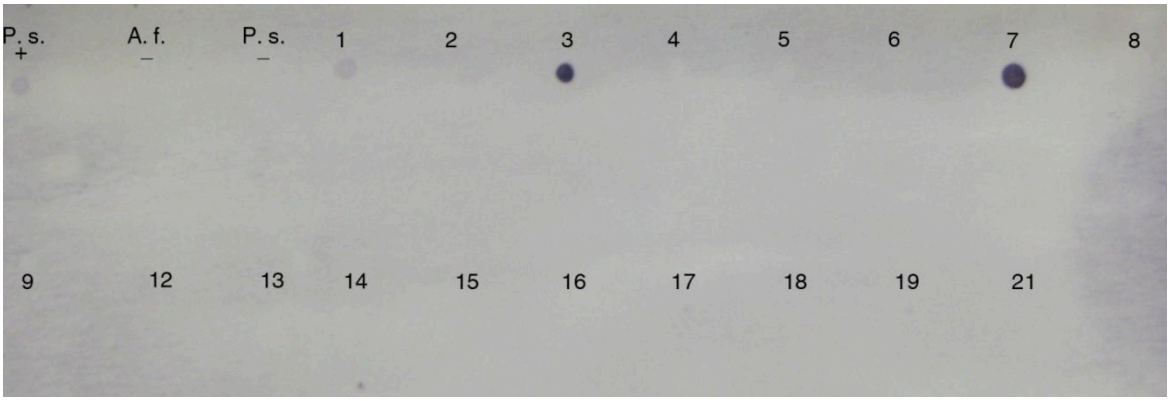

FIG. 14. Hybridization for *P. stutzeri* WM88 *ptxD*. P. s. + is *P. stutzeri* WM88 and a positive control; A. f. – is *A. faecalis* WM2072 and a negative control; P.s. – is *P. stutzeri* WM3617 and a negative control; numbers indicate BS isolates.

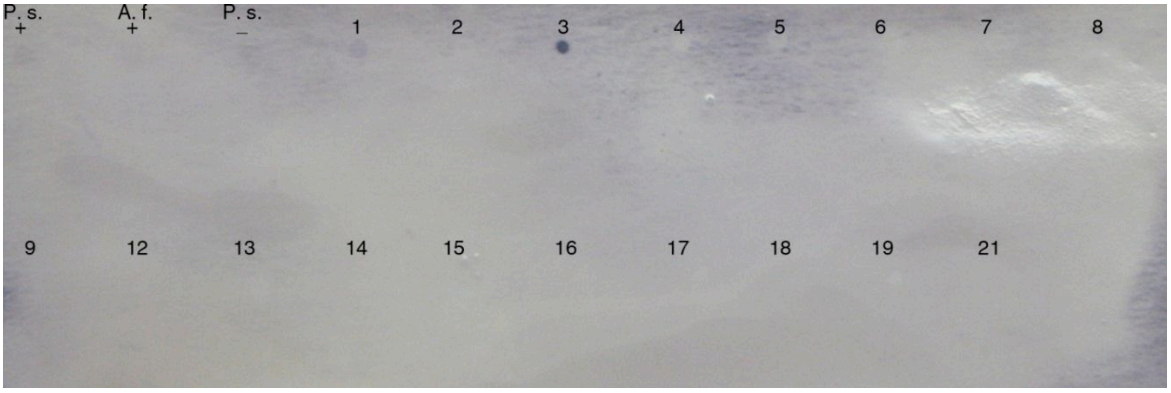

FIG. 15. Hybridization for *htxA*. P. s. + is *P. stutzeri* WM88 and a positive control; A. f. + is *A. faecalis* WM2072 and a positive control; P.s. – is *P. stutzeri* WM3617 and a negative control; numbers indicate BS isolates.

PCR for *ptxD* and *htxA*. PCR using primers for the three known *ptxD*s and one known *htxA* was performed for each isolate (Table 4). Amplified products were the predicted sizes (Table 2). Thirteen of nineteen isolates possessed an amplifiable *htxA* (Fig. 16). *B. elkanii* BS9 was capable of growth on

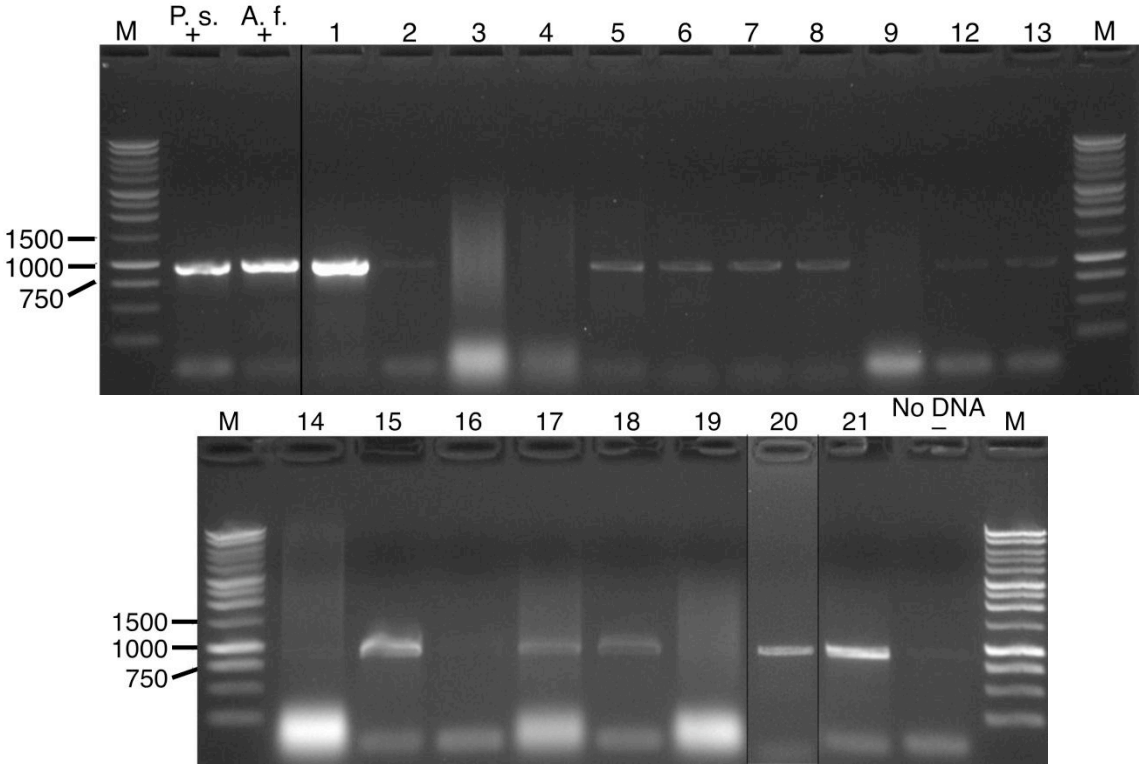

FIG. 16. A 1% TAE agarose gel stained with ethidium bromide for *htxA* PCR products for all isolates. The band for *htxA* is 936 bp. Lane designation: P. s. + is *P. stutzeri* WM88 and a positive control; A. f. + is *A. faecalis* WM2072 and a positive control; numbers indicate BS isolate.

but lacked a detectable *htxA*. *P. stutzeri* BS3 and *Pseudomonas* sp. BS19 were capable of strong growth on Hpt but lacked a detectable *htxA*. Between the three *ptxD* primer sets used, a product of the predicted size was obtained from every isolate (Fig. 17, Fig. 18, Fig. 19, and Fig. 20). PCR for the *X. flavus ptxD* resulted in the amplification of multiple bands. Increasing the annealing temperature resulted in the amplification of one band that was the predicted size but multiple bands were still present (Fig. 19 and Fig. 20). For *V. paradoxus* BS2, *M. luteus* BS12, *Acinetobacter* Acb BS14, and *Streptomyces* sp. BS20, increasing the annealing temperature allowed for the amplification of a single band. Further

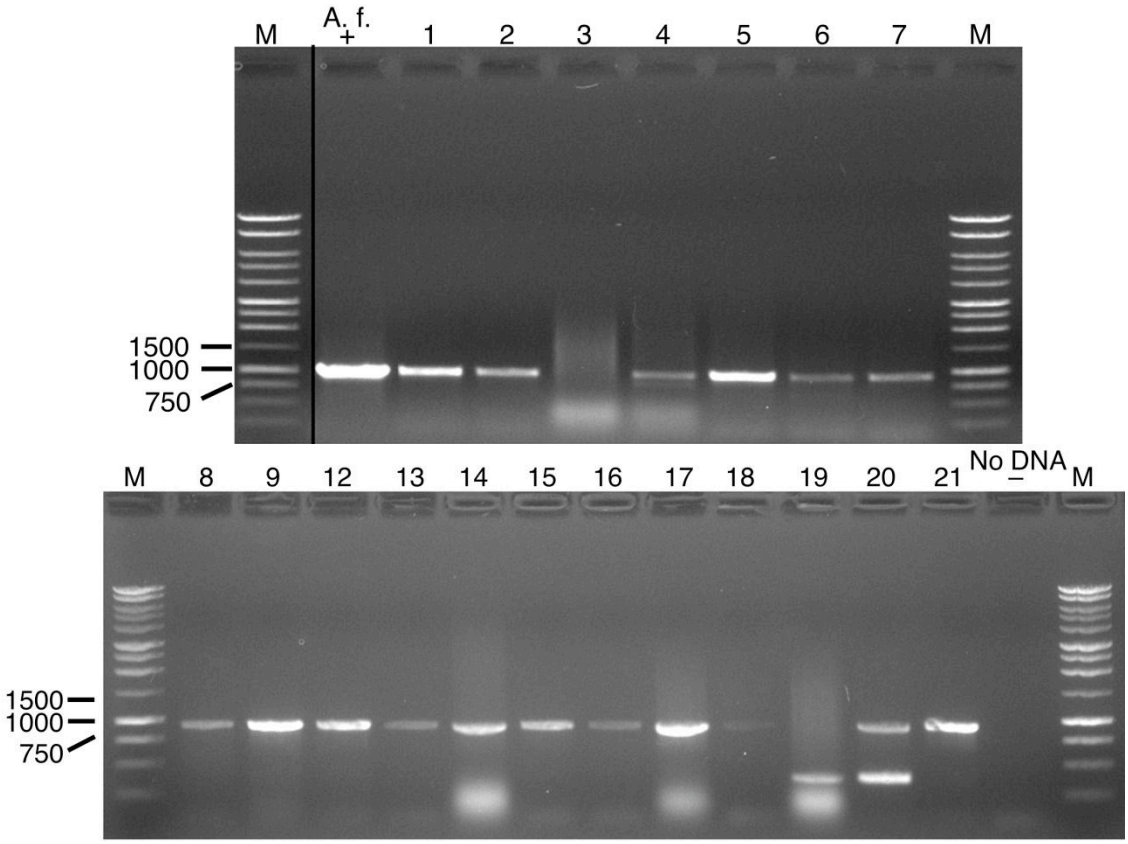

FIG. 17. A 1% TAE agarose gel stained with ethidium bromide for *A. faecalis* WM2072 *ptxD* PCR products for all isolates. The band for *ptxD* is 934 bp. Lane designation: A. f. + is *A. faecalis* positive control; numbers indicate BS isolate.

troubleshooting is required to amplify a single product from the remaining isolates.

Sequencing and alignment of *htxA*. The PCR products from *V. paradoxus* BS5, *V. paradoxus* BS8, *P. fluorescens* BS1, *P. putida* BS15, *P. putida* BS17, *Pseudomonas* sp. BS18, *Pseudomonas* sp. BS21, and *M. luteus* BS12 were sequenced. Sequences obtained allowed the first 798 nucleotides of the 861 nucleotide protein coding sequence to be determined. All *htxA* sequences obtained were 100% identical at the nucleotide and amino acid (266 residues) levels to the *P. stutzeri* WM88 and *A. faecalis* WM2072 *htxA* (data

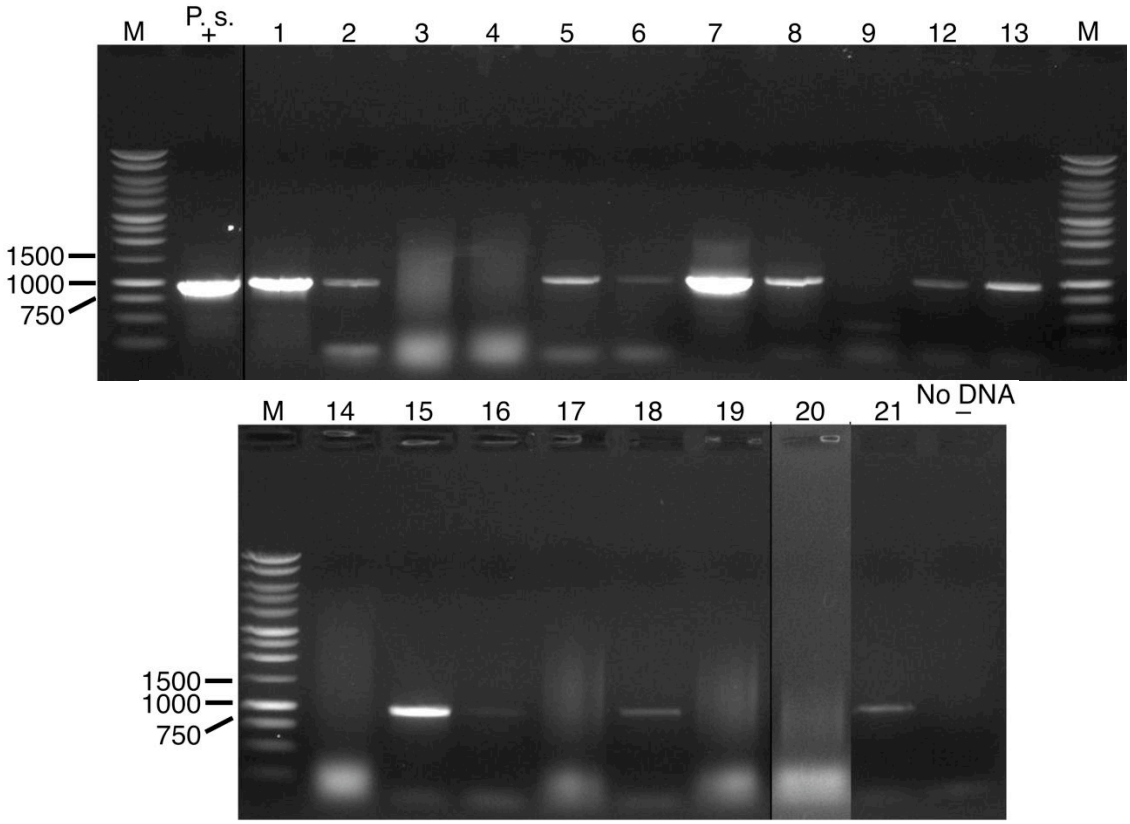

FIG. 18. A 1% TAE agarose gel stained with ethidium bromide for *P. stutzeri* WM88 *ptxD* PCR products for all isolates. The band for *ptxD* is 953 bp. Lane designation: P. s. + is *P. stutzeri* positive control; numbers indicate BS isolate.

not shown).

Sequencing *P. stutzeri* WM88 and *X. flavus* WM2814 *ptxD*.

Sequencing was not performed for the *P. stutzeri* WM88 and *X. flavus* WM2814 *ptxD* products. After numerous successful amplifications with the isolates, PCR repeatedly failed when using the *P. stutzeri* WM88 *ptxD* primer set (PCR was always successful using *P. stutzeri ptxD* with *P. stutzeri* WM88). Numerous troubleshooting steps were attempted including preparing new chromosomal DNA, new GoTaq Green and primer stocks, new PCR cycles, and the addition of DMSO. The only exception was *P. mendocina* BS7, which requires multiple

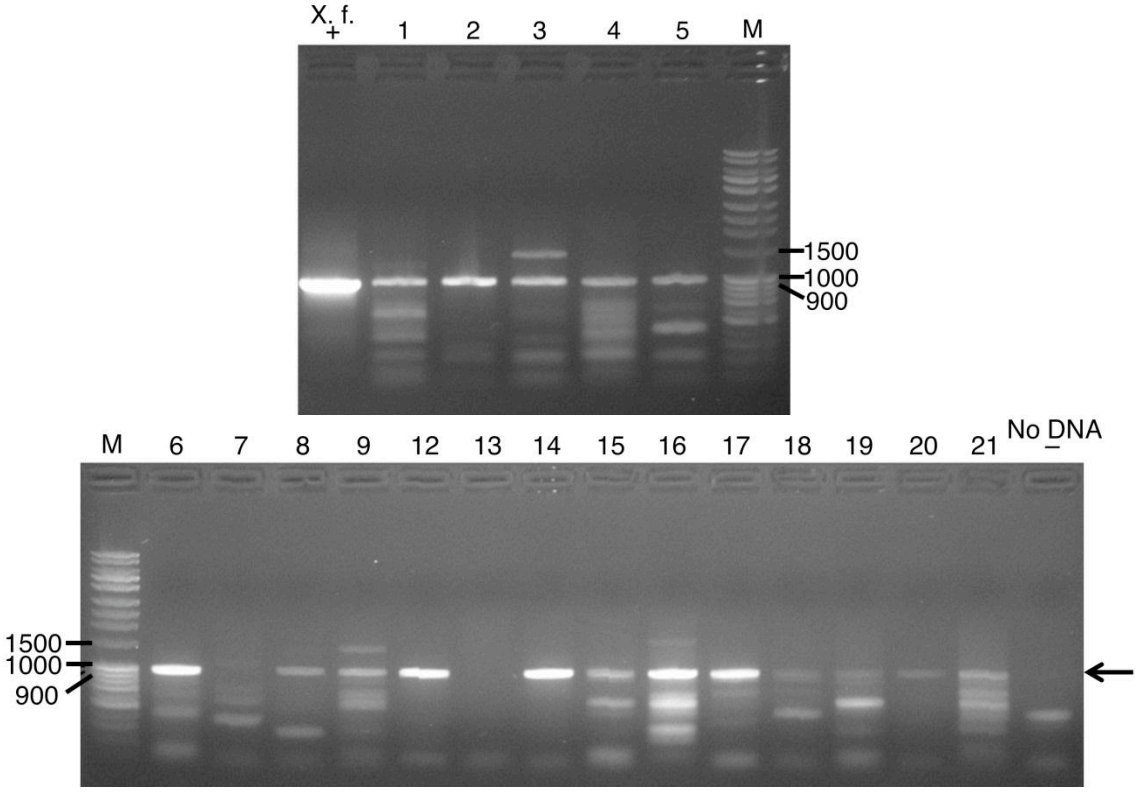

FIG. 19. A 1% TAE agarose gel stained with ethidium bromide for *X. flavus* WM2814 *ptxD* PCR products for all isolates. PCR was run using a one cycle PCR program with an annealing temperature of 57°C. The band for *ptxD* is 962 bp and is indicated by the arrow. Lane designation: X. f. + is *X. flavus* positive control; numbers indicate BS isolate.

amplifications to obtain enough PCR product to sequence.

As stated previously, PCR with *X. flavus* WM2814 *ptxD* was difficult due to the high GC content of the *ptxD* gene (72.8%). At this time, no sequence data have been obtained for the few isolates with single amplifiable bands (*V.* 5 *paradoxus* BS2, *Acinetobacter* Acb BS14, *Streptomyces* sp. BS20, and *M. luteus* BS12) and PCR is still being optimized to determine whether the remaining isolates with multiple bands are true *X. flavus ptxD* products.

Sequencing and alignment of *A. faecalis* WM2072 *ptxD.* The PCR

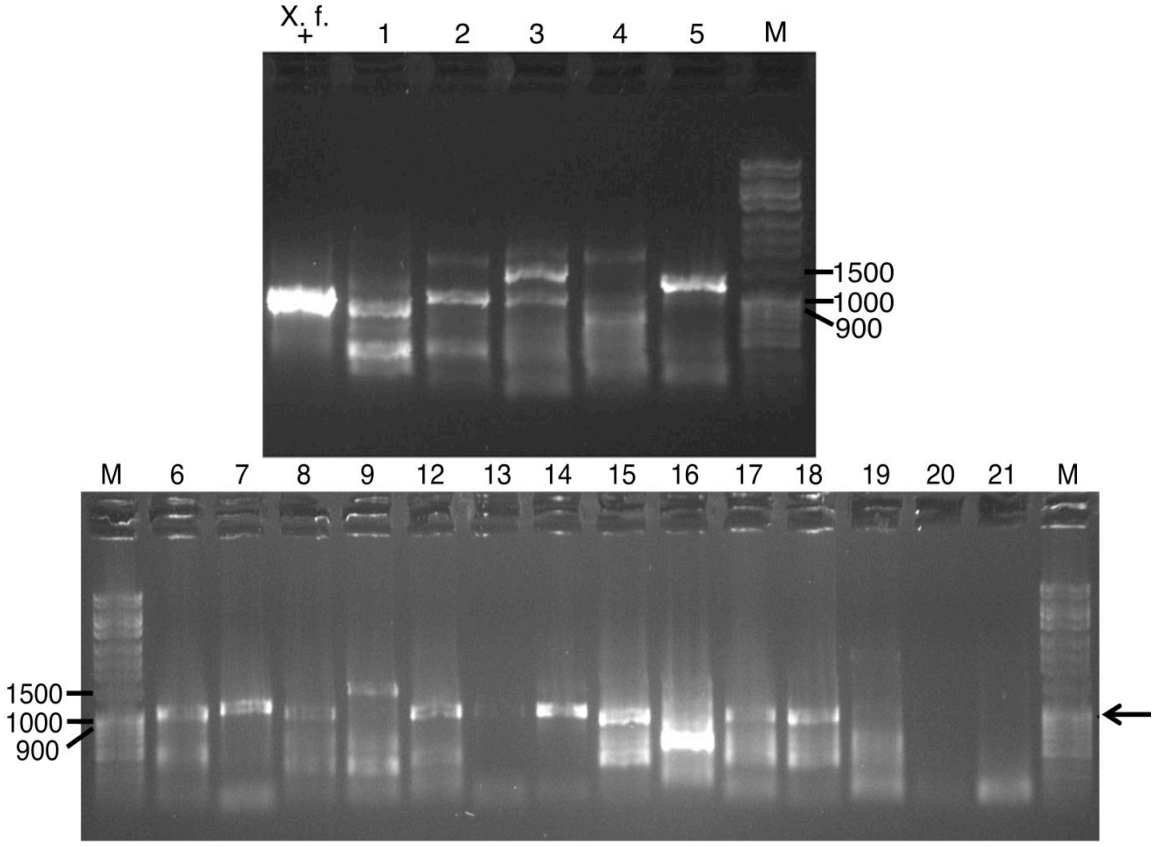

FIG. 20. A 1% TAE agarose gel stained with ethidium bromide for *X. flavus* WM2814 *ptxD* PCR products for all isolates. PCR was run using a two cycle PCR program with annealing temperatures of 55°C 30X and 50°C 10X. The band for *ptxD* is 962 bp and indicated by the arrow. Lane designation: X. f. + is *X. flavus* positive control; numbers indicate BS isolate. A No DNA negative control was clean.

products obtained from *V. paradoxus* BS2, *V. paradoxus* BS5, *V. paradoxus*BS6, *P. fluorescens* BS1, *P. reinekei* BS4, *P. mendocina* BS7, *P. aeruginosa* BS16, *P. putida* BS15, and *P. putida* BS17 were sequenced. Sequences obtained covered 838 nucleotides within the protein coding region. All *ptxD* sequences obtained were 100% identical at the nucleotide and amino acid levels to the *A. faecalis* WM2072 *ptxD* (Fig. 21).

Database sequences. A BLAST search using the *A. faecalis*

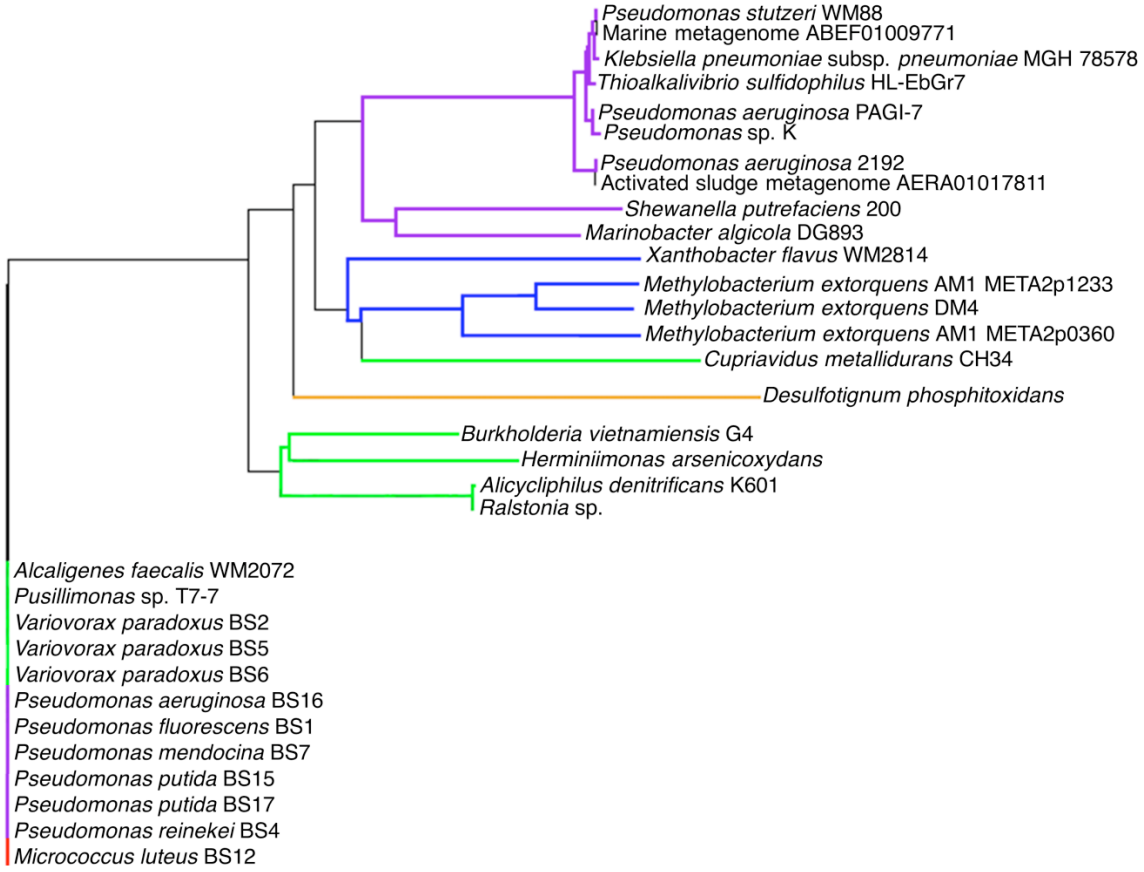

FIG. 21. Phylogenetic tree of *ptxD* amino acid sequences obtained from searching the NCBI databases (see "Database mining" in Methods) and sequencing BS isolate *ptxD* PCR products. All BS isolates were 100% identical to the *A. faecalis* WM2072 *ptxD*. Red lines show Gram-positive bacteria; blue lines show α-Proteobacteria; green lines show β-Proteobacteria; purple lines show γ-Proteobacteria; orange line shows δ-Proteobacteria. Phylogenetic tree created using ClustalW. See Table A2 in Appendix A for a matrix comparison of sequence homologies

WM2072/*P. stutzeri* WM88 *htxA* amino acid sequence revealed a 100% match to a 286 residue region in *Pusillimonas* sp. T7-7 (GenBank accession CP002663). This region is annotated as an alpha-ketoglutarate-dependent hypophosphite dioxygenase. A BLAST search of the entire *A. faecalis* WM2072 *htx-ptx* operon shows this *Pusillimonas* sp. also shares 99% identity across ca. 7362 nucleotides comprising ca. 87% of the *A. faecalis htx-ptx* operon.

A BLAST search using the *A. faecalis* WM2072 *ptxD* amino acid sequence revealed a 100% match to a 333 residue region of *Pusillimonas* sp. T7-7. The *P. stutzeri* WM88 *ptxD* amino acid sequence matched ca. 98% to *P. aeruginosa* 2192 and 99% to *Pseudomonas* sp. K (GenBank accession HQ910240), *K. pneumoniae* MGH 78578 (GenBank accession CP000648), and *Thioalkalivibrio* sp. HL-EbGR7 (GenBank accession CP001339). No matches were found using the *X. flavus* WM2814 *ptxD* sequence.

A BLAST search of the Environmental samples and Whole-genome shotgun read databases using the nucleotide sequences for *htxA* and all three *ptxD* sequences revealed no matches to *htxA.* No novel matches to *A. faecalis* or *X. flavus ptxD* sequences were found in these two databases. Using the *P. stutzeri ptxD* sequence revealed three novel sequence matches that were not found in the Nucleotide collection database: a marine metagenome (GenBank accession ABEF01009771) matched ca. 99%; an activated sludge metagenome (GenBank accession AERA01017811) matched ca. 98%; and *Marinobacter algicola* DG893 (GenBank accession ABCP01000001) matched ca. 66%.

Tracing the evolutionary history of known *ptxD* sequences. The 100% sequence homology of *htxABCD* from *A. faecalis* WM2072, a β-Proteobacteria, and *P. stutzeri* WM88, a γ-Proteobacteria, suggest horizontal transfer occurred and no evolutionary history is clear. The 50% sequence homology of *ptxDE* from *A. faecalis* WM2072, *P. stutzeri* WM88, and *X. flavus* WM2814, an α-Proteobacteria, suggest a long evolutionary history. To obtain more data for the evolutionary history of *ptxD*, 16S rRNA sequences from

organisms identified to have a *ptxD* were aligned (Fig. 22). For those without a published 16S sequence, a substitute sequence (same species but different strain) was used instead. For the two metagenome sequences, no 16S sequence could be found and so are not included in Fig. 22.

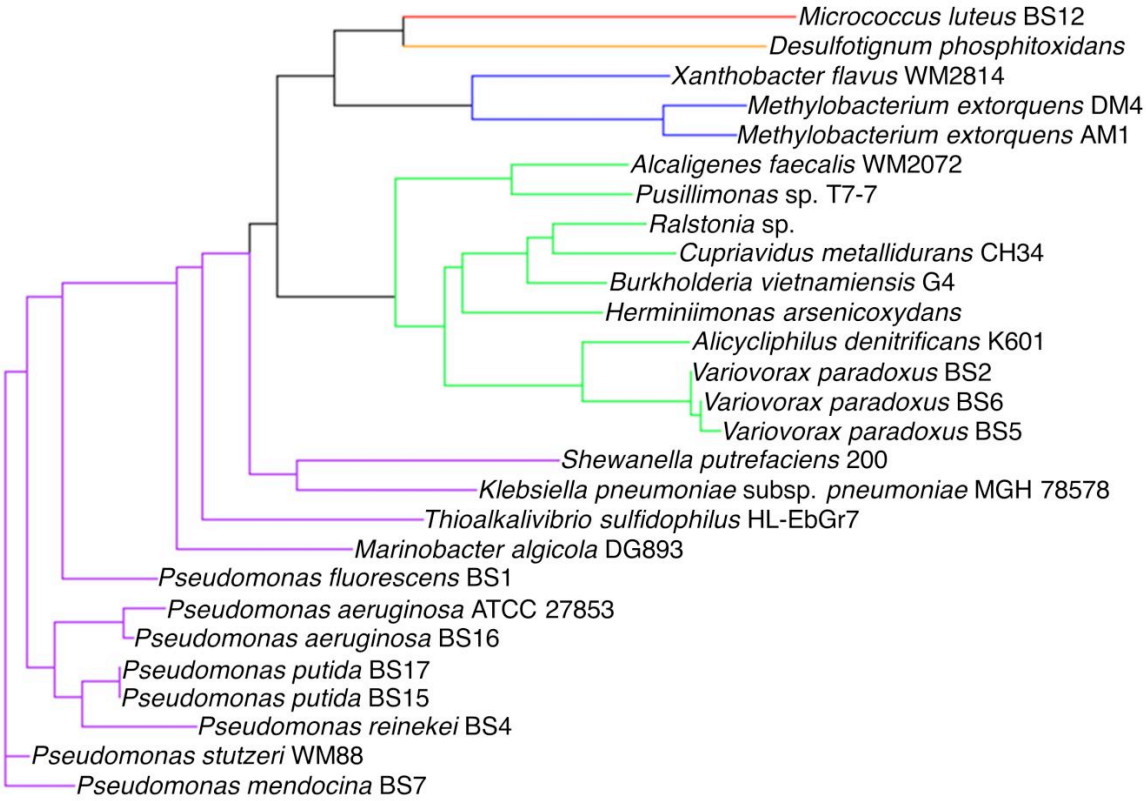

FIG. 22. 16S rRNA phylogenetic tree for bacteria possessing a *ptxD* ortholog (see Fig. 21). Blue lines show α-Proteobacteria; green lines show β-Proteobacteria; purple lines show γ-Proteobacteria; orange line shows δ-Proteobacteria; red line shows the Gram-positive *M. luteus* BS12. Phylogenetic tree created using ClustalW.

The *ptxD* sequences fall into distinct phylogenetic groups with the exception of *Cupriavidus metallidurans* CH34, a β-Proteobacteria with a *ptxD* more similar to those found in the α-Proteobacteria. This suggests horizontal

transfer may have occurred more recently in *C. metallidurans* than in the other β-Proteobacteria. The grouping of *ptxD* sequences obtained in this study from γ-Proteobacteria and the Gram-positive *M. luteus* BS12 suggest potential horizontal transfer that crosses bacterial divisions (68).

CHAPTER IV

DISCUSSION

Most Probable Number for Reduced Phosphorus Oxidizing Bacteria

Research into various aspects of the P cycle, particularly biologically mediated redox of P compounds, has been largely ignored especially when compared to the amount of research still performed on other equally important biogeochemical cycles where bacteria have substantial roles in the redox of nutrients (e.g. nitrogen, sulfur, carbon). This lack of interest and discussion lead me to conclude the apparent consensus held by many outside the field of reduced P biogeochemistry is that RPO, for any purpose (e.g. as an energy or a sole P source), is so rare among environmental bacteria the issue is not a pressing one to thoroughly investigate. My results, however, show RPOB are present in high concentrations regardless of the season, site, or soil characteristics. The high concentration of AEpn oxidizers observed in this study is consistent with previous findings that bacteria able to degrade reduced organic P compounds such as AEpn, are common in the environment, as is the biological production of these phosphonate compounds (42, 63). From these data, it can be concluded that the concentrations of bacteria with the ability to use the reduced P compounds, Hpt, Pt, and AEpn, as a sole source of P are exceedingly high in these common environments.

Unfortunately, bacterial RPO is still in its infancy and as such, there remain numerous fundamental questions that must be answered to obtain a full understanding of P biogeochemistry, or at least one on par with other biogeochemical cycles. For example, the temporal progression for *in situ* oxidation of PH_3 to Pi is not well established. This means the concentrations and biological availability of PH_3, Hpt, and Pt in the environment as well as how the oxidation of PH_3 affects concentrations and availabilities of Hpt and Pt are not established (i.e. is PH_3 immediately oxidized to Pi or is it first oxidized to Hpt, then to Pt, and finally to Pi?). Ultimately this means the effect on the availability of reduced P compounds by bacteria is not established. In addition, biologically relevant concentrations have not been established. As a result, experimental concentrations may be toxic to these populations (discussed below) (21).

In terms of oxidation rates, it is known that as oxidation states decrease, the stability in the presence of oxygen also decreases. Thus, in a typical aerobic environment where there are no apparent consistent sources of inorganic reduced P compounds, it would be expected that Pi and perhaps AEpn would be the predominant P species with Pt, Hpt, and PH_3 ranking somewhere below respectively. In this environment it would be expected the majority of bacteria would use Pi and AEpn with some minority capable of using inorganic reduced P compounds. In an aerobic environment where the availability of any reduced P compound is unknown but quite possibly present (e.g. agricultural areas), it is expected there would be more bacteria capable of using reduced P compounds, particularly if horizontal gene transfer is commonly occurring among

the populations (45, 66, 68). In other words, the P profile of an area should mimic the bacterial profile. However, this idea is dependent upon determining *in situ* concentrations and the rate of oxidation of the reduced P compounds. While site-specific P composition (in soils this will require new or refined detection and quantification methods) and bacterial population profiles are needed to clarify the data in future studies, studies of ammonia-oxidizing bacteria provide potential insight into the possible correlations between P and RPOB profiles. Temporal changes in ammonia-oxidizing bacteria populations resulting from the introduction of ammonia have shown the concentration of ammonia-oxidizing bacteria typically increases when ammonia is introduced (6, 17, 46, 53). This means that concentrations of RPOB may increase when reduced P compounds are introduced.

There is currently little direct evidence for the presence of substantial concentrations of Hpt and Pt in the environment. Pt has been detected in a pristine anaerobic geothermal pool in low concentrations (0.06 μM) (47). In other less pristine environments, Hpt and Pt have not been clearly identified in situ. PH_3, however, has been detected in several anaerobic sites (25). Highly reduced compounds are extremely stable in anaerobic environments, thus it is not surprising that PH_3 has been found in numerous anaerobic environments such as swamps and marshes (8, 16, 19, 25-28, 31, 51, 52). In aerobic environments, however, PH_3 rapidly oxidizes (the half-life can be as few as 5 h) to Pi (69). While it has not been directly demonstrated, presumably when PH_3 oxidizes to Pi, it does so through Hpt and Pt intermediates. Hilton and Robinson (31) found

approximately 70% of PH_3 was detectable after 40 days as Pi. This suggests at least some of the remaining 30% may have been in the form of Hpt and/or Pt. Indeed, Robinson and Bond, in a previous experiment found Hpt and Pt were products of PH_3 oxidation (50). While there are no data available for the half-life of Hpt in soil (59), the half-life of Pt is on the order of a few months (4, 39). From all of this, it might be expected that a higher concentration of RPOB would be found in more anoxic sediments. To explain the high concentrations of bacteria able to oxidize them, it might be suggested that this is the result of the introduction of reduced P compounds into the environment by human activities. To examine the effects of introduced reduced P compounds on the concentrations of bacteria that can oxidize Pt, Hpt and AEpn in the environment, MPN of reduced P oxidizers were determined in samples ranging from pristine to those with extensive agricultural and urban run-off. When the MPN/g from Sed1 (pristine environment) are compared to those from Sed8 (downstream from numerous agricultural areas including one known to use PH_3 and Roundup® as fumigants and pesticides), it is clear that similar numbers of reduced P oxidizers are observed in both sites (Fig. 10). Thus, the high concentrations of bacteria that can carry out RPO in these environments are independent of introduced reduced P compounds. This suggests that reduced P compounds such as Hpt and Pt may indeed be present in the natural environment in significant enough quantities to maintain a high concentration of bacteria that can use them as a P source.

While the MPN data clearly demonstrate RPOB are present, MPN

does not allow for the identification of bacterial species in the original sample and their contribution to RPO. Thus, the diversity of RPOB from the 12 sites is unknown. Identifications (Fig. 13 and Table 4) and preliminary data using direct plating of soil samples onto Hpt, Pt, and AEpn do show diversity among RPOB (data not shown).

While it was assumed that any bacteria capable of growth on the reduced P compounds would also grow on Pi, there are limitations: (1) MPN provides only an estimate of culturable bacteria, which is further compounded due to the small percentage of environmental bacteria that are culturable under laboratory conditions and the highly selective nature of the media used in this study. (2) MPN analysis assumes an even distribution of bacteria. That is, it is assumed the same bacteria present in the Pi media are also present in Hpt, Pt, and AEpn media. (3) Environmental concentrations of the P compounds (including Pi) may be significantly lower than experimental concentrations. This may result in experimental concentrations (including Pi) being toxic to some or many environmental bacteria (21). (4) The concentrations of the reduced P compounds in certain environments may be higher than Pi making growth on reduced P compounds easier. (5) The bacteria cultured may have uncharacterized mechanisms that allow for growth on Hpt and Pt that would lead some species outgrowing others. Currently all pathways characterized for RPO requires Hpt to be oxidized to Pt and then to Pi (45, 67, 68). A novel pathway could allow for more efficient oxidation of the reduced P compounds resulting in relatively faster growth.

Genetic Analyses for *htxA* and *ptxD*

The aforementioned hypothesis that the P profile (e.g. concentrations of reduced P compounds) and microbial profile (e.g. concentrations of reduced P oxidizers) should positively correlate is not unreasonable and is consistent with microbial genetics that repeatedly shows nonessential genes encoding for nonessential functions are readily lost (14). *P. stutzeri* WM88, *A. faecalis* WM2072, and *X. flavus* WM2814 all have entire operons devoted to transporting and metabolizing reduced P compounds with regulatory genes often included and which are induced in response to Pi starvation (45, 66-68). While it is currently speculation, it logically follows that the reduced P compounds Hpt, Pt, and AEpn must be present in natural environments in concentrations that are biologically useful to these organisms and populations, including unculturable populations, to keep selective pressure on the local bacterial populations to gain, retain, and maintain some form of a functional RPO metabolic pathway. The genetic data in this study supports this conclusion.

All isolates appear to have some ortholog of *ptxD*. However, two were incapable of growth on Pt (*M. luteus* BS12, *P. putida* BS17) and six were capable of moderate growth on Pt (*P. stutzeri* BS3, *P. reinekei* BS4, *V. paradoxus* BS5, *V. paradoxus* BS6, *P. aeruginosa* BS16, *Pseudomonas* sp. BS21). The presence of *ptxD* does not mean the isolate possesses a fully functional and complete *ptx* operon. Without complete sequence data for the entire *ptxD* gene, a truncated or mutated *ptxD* cannot be ruled out. In addition, a truncated or mutated *ptx* operon could result in poor or no transporting of Pt.

There were notable differences between PCR and hybridization results. For *P. stutzeri* WM88 *ptxD* and *htxA*, only *P. fluorescens* BS1 and *P. mendocina* BS7 were positive with both PCR and hybridization. For *htxA*, *P. stutzeri* BS3 was negative with PCR and positive for hybridization yet grows well on Hpt. For *P. stutzeri* WM88 *ptxD*, *P. stutzeri* BS3 (moderate growth on Pt), *P. putida* BS17 (no growth on Pt), and *Pseudomonas* sp. BS19 (strong growth on Pt) were negative with PCR and positive with hybridization. Twelve isolates were positive with PCR and negative with hybridization. Five were negative with both PCR and hybridization.

There are three possible explanations as to why an isolate would be positive for one (PCR or hybridization) and negative with the other. One explanation may have to do with the homology of the primer region versus homology of the entire gene. The primers used in PCR are ca. 20 nucleotides while the genes used in hybridization are between ca. 850 to 950 bp. It is possible there are mutations in the primer regions that prevent amplification but not hybridization. It also may be that non-specific binding (hybridization) or amplification (PCR) is occurring.

Without sequence data for the *P. stutzeri* WM88 *ptxD, X. flavus* WM2814 *ptxD,* or the remaining *htxA* PCR products, it cannot be determined whether non-specific amplification is occurring during PCR. Finally, the *htxA* forward primer binds 75 nt upstream of the start codon. It may be that *P. stutzeri* BS3 (negative PCR, positive hybridization) has mutations in this upstream region that prevents primer binding. Ultimately, this may mean *P. stutzeri* BS3 has an

htxA that is less than 100% identical to the *P. stutzeri* WM88 and *A. faecalis* WM2072 *htxA*. Without in-depth genetic analyses of those with negative PCR and positive hybridization, I cannot determine to what region in the chromosome *htxA* is hybridizing. Further study, especially for those positive with hybridization, negative with PCR, and capable of strong growth on the respective reduced P compound, is required.

There are two possible explanations for those with no detectable *htxA* (negative with PCR and hybridization) capable of oxidizing Hpt (*Pseudomonas* sp. BS19, *Acinetobacter* Acb BS14, *B. elkanii* BS9). These bacteria may have novel Hpt oxidation pathways. Alternatively, they may possess genes similar to the *X. flavus* WM2814 *htxXY*.

Sequence analysis showed all *htxA* PCR products were 100% identical at the amino acid level to the previously characterized *P. stutzeri* WM88 and *A. faecalis* WM2072 *htxA*. This much homology between genes found in bacteria isolated from regions separated by a great geographic distance seems suspicious (*P. stutzeri* WM88 and *A. faecalis* WM2072 were isolated from Illinois; isolates from this study are from Oregon). Contamination can be ruled out since some isolates did not give a PCR product and the negative (no DNA) control was always clean. Sequence errors, however, cannot be ruled out since the Taq polymerase used in this study is not a high-fidelity polymerase.

It is important to note *Pusillimonas* sp. T7-7, an isolate from the Bohai Sea in China, has not only an *htxA* that matches 100% with *htxA* from *P. stutzeri* WM88 and *A. faecalis* WM2072, but 87% (7362 of 8193 nucleotides) of the entire

A. faecalis WM2072 *htx-ptx* operon shares 99% sequence homology to a region in *Pusillimonas* sp. T7-7. Given 87% of a nearly 8.2-kb region appears to be so strongly conserved in isolates from Illinois and China strongly suggests finding a region of ca. 0.9-kb conserved in isolates from Oregon is not improbable.

All of the *ptxD* sequences were 100% identical at the amino acid level to the *A. faecalis* WM2072 *ptxD*. This was surprising since, unlike with *htxA*, three *ptxD* orthologs have been identified and each are 50% identical at the amino acid level. Contamination was not an issue as some isolates did not give a PCR product and the negative control (no DNA) was always clean. The PCR products from *P. fluorescens* BS1, *V. paradoxus* BS5, and *M. luteus* BS12 were resequenced. Unfortunately, the sequence data was incomplete. The incomplete data did show that for *P. fluorescens* BS1, nucleotides 72-262 and 638-900 and for *V. paradoxus* BS5, nucleotides 92-298 and 498-878 were 100% identical to *A. faecalis*. More sequence data was available for *M. luteus* BS12 and showed nucleotides 70-888 were 100% identical to *A. faecalis*.

Without sequence data from *P. stutzeri* WM88 and *X. flavus* WM2814 *ptxD* PCR products, it is extremely difficult to completely interpret the *ptxD* data and many questions remain to be answered. What is the actual distribution of each *ptxD* gene in the natural environment? Do most of the isolates have an *A. faecalis* WM2072 *ptxD*? Do some of the isolates have multiple *ptxD* genes or am I amplifying *P. stutzeri* WM88 *ptxD* genes with the *A. faecalis ptxD* primer set? It does seem unlikely, given the difficulty I have encountered when using *X. flavus* WM2814 *ptxD* primers on my isolates, that I am amplifying *X. flavus ptxD* with

either *P. stutzeri* or *A. faecalis ptxD* primers. It would seem much more likely I would amplify *P. stutzeri* or *A. faecalis ptxD* with *X. flavus ptxD* primers.

The increasing ease of genome sequencing will help to answer the distribution question for both *ptxD* and *htxA*. Five sequences (*Bradyrhizobium* sp. BTAi1, GenBank accession CP000494.1; *Roseibium* sp. TrichSKD4, GenBank accession GL476319; *Pantoea ananatis* AJ13355, GenBank accession AP012032; *P. ananatis* LMG 20103, GenBank accession CP001875; *Burkholderia rhizoxinica* HKI 454, GenBank accession FR687359) in the NCBI nucleotide database are annotated as *htxA*, HtxA-like, hypophosphite dioxygenase, or putative alpha-ketoglutarate-dependent hypophosphite dioxygenase. However, aligning these sequences with empirically known *htxA* sequences showed these were not *htxA* genes (amino acid similarities to *htxA* ranged from 16-44%) (See Table A3 in Appendix A for a matrix comparison of sequence homologies**).** The percent similarities, as well as the presence of conserved motifs (specifically the H-X-D-X motif), suggest these sequences are part of the dioxygenase superfamily, not the *htxA* protein family.

Searching the terms "*ptxD*" and "phosphite dehydrogenase" in the BLAST nucleotide database, as well as including bacteria previously determined to have *ptxD,* resulted in 17 unique bacteria. Four bacteria found using *P. stutzeri* WM88 were 99-100% identical indicating the *P. stutzeri ptxD* is strongly conserved. Three bacteria found using *A. faecalis* WM2072 *ptxD* were ca. 65% and 100% identical indicating more diversity. No bacteria were found using *X. flavus* WM2814 that covered at least 80% of the query sequence.

The database results showing more bacteria match the *P. stutzeri ptxD* suggest this ortholog may be more common in the environment. However, the sequence analysis results presented here suggest the *A. faecalis ptxD* ortholog may be more common. Since isolates identified in this study came from the same environmental sample, it is entirely possible horizontal transfer events occurred among the sample population. There is evidence *ptxD* has been horizontally transferred between the β- and γ-Proteobacteria classes (68). While horizontal transfer is not uncommon between Gram-negative and Gram-positive bacteria, it has not been demonstrated for *ptxD*. This is because *ptxD* had only been identified in Gram-negative bacteria.

Broader implications. The potential impacts of bacterial RPO are great and include the degree to which P is available, obtaining a fuller understanding of bioremediation, as well as developing and maintaining effective agricultural practices. In terms of P availability, there is concern that P, specifically Pi, is not a renewable resource (32). The influence RPOB have on P availability will remain unknown until further multi-disciplinary research is performed on a wide range of environments. The impact on bioremediation, especially with the increasing popularity of reduced P compounds like Pt in fertilizers and as a treatment for Sudden Oak Death (22, 37), may not be as clear but is still important given the unknown state of biotic and abiotic oxidation of reduced P compounds. Currently, the assumption is when Pt is applied to an area it rapidly oxidizes to Pi and thus there are little or no ramifications from using Pt. There are at least four problems with this idea. First, only a handful of

studies have shown the oxidation rate of Pt *in situ* (estimated half-life is on the order of a few months) (4, 39) but, this rate is likely dependent on a multitude of site-specific or region-specific factors. Thus, not only are studies required to determine the rate of Pt oxidation under various environmental conditions, also required are studies using equipment that can detect small biologically useful concentrations of Pt. Second, the total effects on the surrounding environment from the introduction of Pt are unknown but given bacteria can oxidize Pt to Pi, there are likely some effects. Third, at the very least, assuming Pt rapidly oxidizes to Pi, the introduction of Pt is introducing high amounts of Pi, which can lead to numerous ecological problems (e.g. eutrophication, run-off creating algal plumes and dead zones) (12, 13, 18). Finally, there may be agricultural impacts. The presence of reduced P compounds in fertilizers and herbicides/pesticides may lead to rhizobacterial populations and plants competing for these compounds. The results could be ineffective fertilizers or toxic amounts of P compounds (reduced and Pi) that may affect rhizobacterial populations and, directly or indirectly, plant populations.

Despite the increasing number of reports demonstrating the oxidation of reduced P compounds by bacteria, this biological phenomenon continues to be largely overlooked in the current description of P cycling in the environment, perhaps due to a lack of understanding of the environmental significance of this activity. In an effort to further investigate the contribution of bacterial RPO to a global P redox cycle in an environmental context, I sought to quantify microbial RPO in the environment and the genes used. The ubiquity of RPOB and

oxidation genes indicates the apparent importance of this activity in the environment, and provides indirect evidence that these compounds may be important sources of P for bacteria. Finally, these data indicate that our current understanding of P flux and transformation through ecosystems is incomplete. Additional investigations of this activity and its impact on the environment are critical to our understanding of P cycling and availability, which is essential for effective P conservation efforts (23), understanding nutrient availability, and improving agricultural and bioremediation practices. In total, these data provide very strong evidence for the importance of RPO by bacteria in the environment and their roles in altering available Pi levels (Fig. 23). In addition, sufficient evidence is provided to warrant further investigation of bacterial oxidation of reduced P compounds and investigation of the roles bacterially mediated P redox play in P cycling and biogeochemistry.

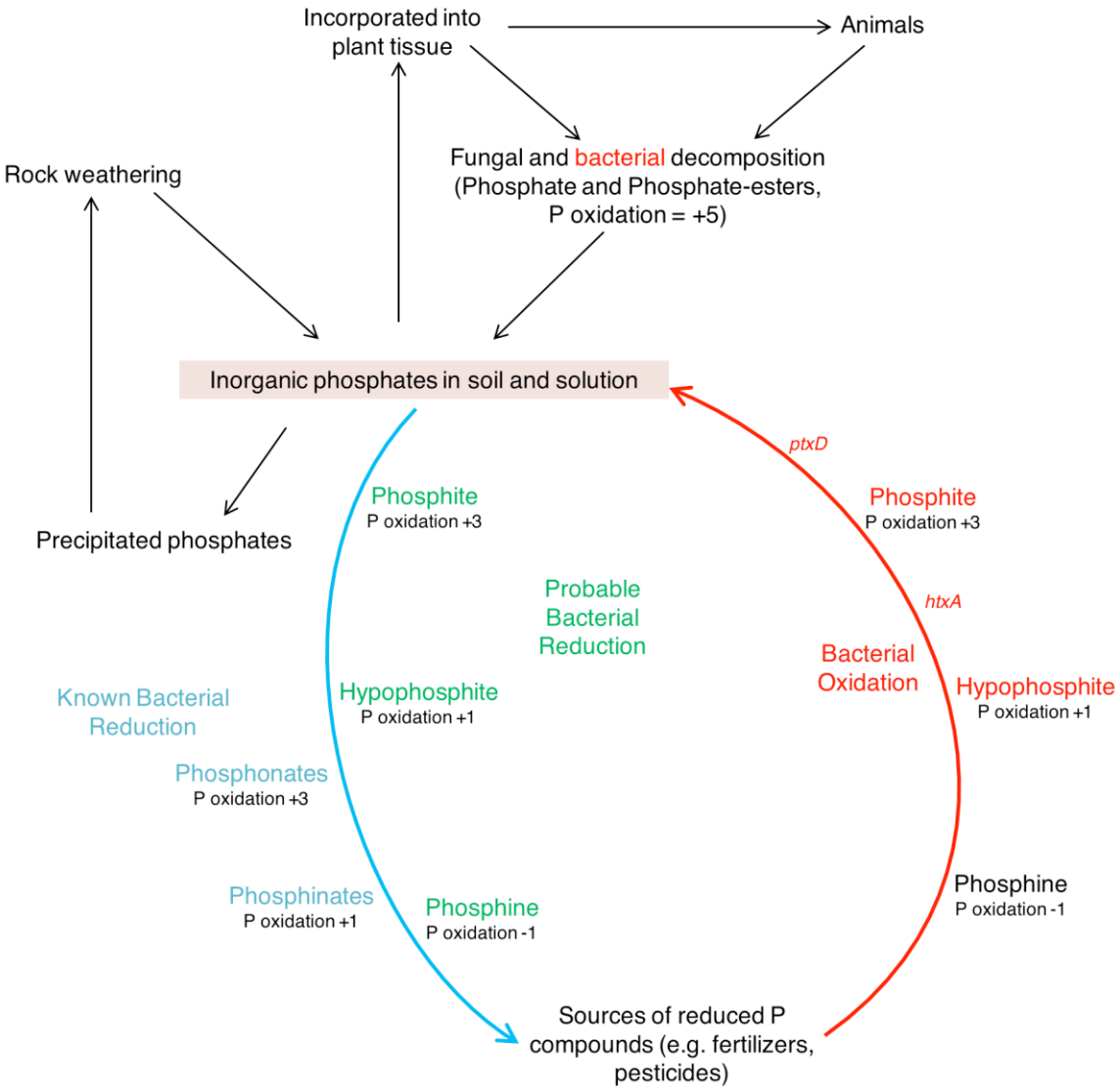

FIG. 23. A more accurate P cycle. By combining the results of this study with results from studies over the last 100 years, a more accurate P cycle can be drawn. Known point sources of reduced P compounds (PH_3, Hpt, and Pt) include fertilizers, pesticides, and industrial processes. Known bacterial oxidation via the genes *htxA* and *ptxD* are shown in red. Known bacterial reduction of phosphonates and phosphinates are shown in blue. While natural sources of PH_3, Hpt, or Pt are still unclear, abiotic reduction has been demonstrated (27) and biotic reduction is possible (51). Possible bacterial reduction is shown in green.

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APPENDIX A

TABLE A1. Pairwise sequence alignment scores for the 16S rRNA sequences of the 19 bacterial isolates identified in this study. Pairwise alignment scores, in addition to phylogenetic trees, can be used to approximate how closely related certain species or genera are to each other.

	B. elkanii BS9	*V. paradoxus* BS2	*V. paradoxus* BS5	*V. paradoxus* BS6	*V. paradoxus* BS8	*Acinetobacter* Acb BS13	*Acinetobacter* Acb BS14	*P. aeruginosa* BS16	*P. fluorescens* BS1	*P. mendocina* BS7	*P. putida* BS15	*P. putida* BS17	*P. reinekei* BS4	*P. stutzeri* BS3	*Pseudomonas* sp. BS18	*Pseudomonas* sp. BS19	*Pseudomonas* sp. BS21	*M. luteus* BS12	*Streptomyces* sp. BS20
Bradyrhizobium elkanii BS9	---	82	80	81	80	81	77	81	77	80	79	79	78	81	78	83	82	77	77
Variovorax paradoxus BS2	82	---	99	100	100	78	80	81	81	82	80	81	81	82	79	82	81	79	79
Variovorax paradoxus BS5	80	99	---	99	98	79	76	81	78	81	80	80	81	81	76	81	80	76	78
Variovorax paradoxus BS6	81	100	99	---	99	79	77	81	79	81	80	81	81	82	77	81	81	77	79
Variovorax paradoxus BS8	80	100	98	99	---	79	77	81	79	81	78	78	79	81	78	81	81	77	77
Acinetobacter Acb BS13	81	78	79	79	79	---	98	84	85	85	85	86	86	85	83	86	87	78	79
Acinetobacter Acb BS14	77	80	76	77	77	98	---	83	86	82	82	82	82	85	85	86	85	76	74
Pseudomonas aeruginosa BS16	81	81	81	81	81	84	83	---	93	95	96	96	94	95	93	95	95	77	80
Pseudomonas fluorescens BS1	77	81	78	79	79	85	86	93	---	93	93	93	91	96	97	99	98	75	75
Pseudomonas mendocina BS7	80	82	81	81	81	85	82	95	93	---	96	95	93	97	93	97	96	77	79

TABLE A1 (continued)

	B. elkanii BS9	*V. paradoxus* BS2	*V. paradoxus* BS5	*V. paradoxus* BS6	*V. paradoxus* BS8	*Acinetobacter* Acb BS13	*Acinetobacter* Acb BS14	*P. aeruginosa* BS16	*P. fluorescens* BS1	*P. mendocina* BS7	*P. putida* BS15	*P. putida* BS17	*P. reinekei* BS4	*P. stutzeri* BS3	*Pseudomonas* sp. BS18	*Pseudomonas* sp. BS19	*Pseudomonas* sp. BS21	*M. luteus* BS12	*Streptomyces* sp. BS20
Pseudomonas putida BS15	79	80	80	80	78	85	82	96	93	96	---	100	96	96	95	96	96	77	80
Pseudomonas putida BS17	79	81	80	81	78	86	82	96	93	95	100	---	96	96	96	96	96	77	80
Pseudomonas reinekei BS4	78	81	81	81	79	86	82	94	91	93	96	96	---	94	92	97	96	76	78
Pseudomonas stutzeri BS3	81	82	81	82	81	85	85	95	96	97	96	96	94	---	94	96	96	80	81
Pseudomonas sp. BS18	78	79	76	77	78	83	85	93	97	93	95	96	92	94	---	96	94	78	77
Pseudomonas sp. BS19	83	82	81	81	81	86	86	95	99	97	96	96	97	96	96	---	100	78	80
Pseudomonas sp. BS21	82	81	80	81	81	87	85	95	98	96	96	96	96	96	94	100	---	77	79
Micrococcus luteus BS12	77	79	76	77	77	78	78	77	75	77	77	77	76	80	78	78	77	---	92
Streptomyces sp. BS20	77	79	76	79	77	79	74	80	75	79	80	78	78	81	77	80	79	92	---

TABLE A2. Pairwise sequence alignment scores for the *ptxD* sequences referenced in this study. The alignment scores for *Pusillimonas* sp. T7-7, *V. paradoxus* BS2, *V. paradoxus* BS5, *V. paradoxus* BS6, *P. aeruginosa* BS16, *P. fluorescens* BS1, *P. mendocina* BS7, *P. putida* BS15, *P. putida* BS17, *P. reinekei* BS4, and *M. luteus* BS12 are not included in this table since they were all 100% identical to the *A. faecalis* WM2072 *ptxD* sequence. Pairwise alignment scores, in addition to phylogenetic trees, can be used to approximate how closely related the sequences are to each other.

	Activated sludge	*A. faecalis* WM2072	*A. denitrificans* K601	*B. vietnamiensis* G4	*C. metallidurans* CH34	*D. phosphitoxidans*	*H. arsenicoxydans*	*K. pneumoniae* MGH 78578	Marine metagenome	*M. algicola*	*M. extorquens* AM1 META2p0360	*M. extorquens* AM1 META2p1233	*M. extorquens* DM4	*P. aeruginosa* 2192	*P. aeruginosa* PAGI-7	*Pseudomonas* sp. K	*P. stutzeri* WM88	*Ralstonia* sp. 5-7-47FAA	*S. putrefaciens* 200	*T. sulfidophilus* HL EbGr-7	*X. flavus* WM2814
Activated sludge AERA01017811	---	53	52	52	45	39	55	97	97	64	54	53	51	100	96	96	97	53	59	96	53
Alcaligenes faecalis WM2072	53	---	62	64	44	40	59	52	52	54	49	48	50	53	53	52	52	62	51	52	49
Alicycliphilus denitrificans K601	52	62	---	70	47	42	65	53	53	56	48	52	52	52	54	53	53	100	53	53	51
Burkholderia vietnamiensis G4	52	64	70	---	42	39	67	52	52	53	50	49	50	52	53	53	52	70	50	53	50
Cupriavidus metallidurans CH34	45	44	47	42	---	32	43	45	45	44	49	51	52	45	45	44	45	47	45	45	47
Desulfotignum phosphitoxidans	39	40	42	39	32	---	39	39	39	37	36	36	37	39	38	38	39	42	36	39	34

TABLE A2 (continued)

	Activated sludge	*A. faecalis* WM2072	*A. denitrificans* K601	*B. vietnamiensis* G4	*C. metallidurans* CH34	*D. phosphitoxidans*	*H. arsenicoxydans*	*K. pneumoniae* MGH 78578	Marine metagenome	*M. algicola*	*M. extorquens* AM1 META2p0360	*M. extorquens* AM1 META2p1233	*M. extorquens* DM4	*P. aeruginosa* 2192	*P. aeruginosa* PAGI-7	*Pseudomonas* sp. K	*P. stutzeri* WM88	*Ralstonia* sp. 5-7-47FAA	*S. putrefaciens* 200	*T. sulfidophilus* HL EbGr-7	*X. flavus* WM2814
Herminiimonas arsenicoxydans	55	59	65	67	43	39	---	55	55	53	50	50	50	55	55	55	55	65	51	55	50
Klebsiella pneumoniae MGH 78578	97	52	53	52	45	39	55	---	99	64	55	52	51	97	98	97	99	53	60	98	53
Marine metagenome ABER01009771	97	52	53	52	45	39	55	99	---	64	55	52	51	97	98	98	100	53	60	99	53
Marinobacter algicola	64	54	56	53	44	37	53	64	64	---	54	54	54	64	64	64	64	56	66	64	51
Methylobacterium extorquens AM1 META2p0360	54	49	48	50	49	36	50	55	55	54	---	73	71	54	55	54	55	48	49	55	54
Methylobacterium extorquens AM1 META2p1233	53	48	52	49	51	36	50	52	52	54	73	---	84	53	53	52	52	52	47	52	54
Methylobacterium extorquens DM4	51	50	52	50	52	37	50	51	51	54	71	84	---	51	52	52	51	52	47	52	54
Pseudomonas aeruginosa 2192	100	53	52	52	45	39	55	97	97	64	54	53	51	---	96	96	97	53	59	96	53

TABLE A2 (continued)

	Activated sludge	*A. faecalis* WM2072	*A. denitrificans* K601	*B. vietnamiensis* G4	*C. metallidurans* CH34	*D. phosphitoxidans*	*H. arsenicoxydans*	*K. pneumoniae* MGH 78578	Marine metagenome	*M. algicola*	*M. extorquens* AM1 META2p0360	*M. extorquens* AM1 META2p1233	*M. extorquens* DM4	*P. aeruginosa* 2192	*P. aeruginosa* PAGI-7	*Pseudomonas* sp. K	*P. stutzeri* WM88	*Ralstonia* sp. 5-7-47FAA	*S. putrefaciens* 200	*T. sulfidophilus* HL EbGr-7	*X. flavus* WM2814
Pseudomonas aeruginosa PAGI-7	96	53	54	53	45	38	55	98	98	64	55	53	52	96	---	99	98	54	60	99	53
Pseudomonas sp. K	96	52	53	53	44	38	55	97	98	64	54	52	52	96	99	---	98	53	60	98	53
Pseudomonas stutzeri WM88	97	52	53	52	45	39	55	99	100	64	55	52	51	97	98	98	---	53	60	99	53
Ralstonia sp. 5-7-47FAA	53	62	100	70	47	42	65	53	53	56	48	52	52	53	54	53	53	---	53	54	51
Shewanella putrefaciens 200	59	51	53	50	45	36	51	60	60	66	49	47	47	59	60	60	60	53	---	61	46
Thioalkalivibrio sulfidophilus HL EbGr-7	96	52	53	53	45	39	55	98	99	64	55	52	52	96	99	98	99	54	61	---	53
Xanthobacter flavus WM2814	53	49	51	50	47	34	50	53	53	51	54	54	54	53	53	53	53	51	46	53	---

TABLE A3. Pairwise sequence alignment scores for the *htxA* sequences identified or referenced in this study. Pairwise alignment scores, in addition to phylogenetic trees, can be used to approximate how closely related the sequences are to each other. Sequences with scores greater than 50% are considered to be in the *htxA* family. Sequences with scores lower than 50% are considered to be in the 2-oxoglutarate-dependent dioxygenase superfamily.

	A. faecalis WM 2072	*Bradyrhizobium* sp. BTAi1	*B. rhizoxinixa* HKI	*M. luteus* BS12	*P. ananatis*[a]	*P. fluorescens* BS1	*P. putida* BS15	*P. putida* BS17	*Pseudomonas* sp. BS18	*Pseudomonas* sp. BS21	*P. stutzeri* WM88	*Pusillimonas* sp. T7-7	*Roseibium* sp.	*V. paradoxus* BS5	*V. paradoxus* BS8
Alcaligenes faecalis WM 2072	---	44	16	100	25	100	100	100	100	100	100	100	35	100	100
Bradyrhizobium sp. BTAi1	44	---	18	44	28	44	44	44	44	44	44	44	35	44	44
Bradyrhizobium rhizoxinixa HKI 454	16	18	---	16	22	16	16	16	16	16	16	16	15	16	16
Micrococcus luteus BS12	100	44	16	---	25	100	100	100	100	100	100	100	34	100	100
Pantoea ananatis[a]	25	28	22	25	---	25	25	25	25	25	25	25	24	25	25
Pseudomonas fluorescens BS1	100	44	16	100	25	---	100	100	100	100	100	100	34	100	100
Pseudomonas putida BS15	100	44	16	100	25	100	---	100	100	100	100	100	34	100	100
Pseudomonas putida BS17	100	44	16	100	25	100	100	---	100	100	100	100	34	100	100
Pseudomonas sp. BS18	100	44	16	100	25	100	100	100	---	100	100	100	34	100	100
Pseudomonas sp. BS21	100	44	16	100	25	100	100	100	100	---	100	100	34	100	100
Pseudomonas stutzeri WM88	100	44	16	100	25	100	100	100	100	100	---	100	35	100	100
Pusillimonas sp. T7-7	100	44	16	100	25	100	100	100	100	100	100	---	35	100	100
Roseibium sp. TrichSKD4	35	35	15	34	24	34	34	34	34	34	35	35	---	34	34
Variovorax paradoxus BS5	100	44	16	100	25	100	100	100	100	100	100	100	34	---	100
Variovorax paradoxus BS8	100	44	16	100	25	100	100	100	100	100	100	100	34	100	---

[a] Two strains were found in the NCBI database (AJ13355 and LMG 20103) but the annotated region of interest was 100% identical in both strains.