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Santa Barbara

Exploring the Phosphorus Redox Biogeochemical Cycle with Phosphite

A dissertation submitted in partial satisfaction of the  
requirements for the degree Doctor of Philosophy  
in Chemistry

by

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December 2024

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November 2024

Exploring the Phosphorus Redox Biogeochemical Cycle with Phosphite

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by

Clara Addington Bailey



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## ABSTRACT

### Exploring the Phosphorus Redox Biogeochemical Cycle with Phosphite

by

Clara Addington Bailey

Phosphorus is most commonly found in its fully oxidized state ( $P^{+5}$ ) of phosphate and phosphate esters, which are incorporated into biomolecules such as nucleotides, nucleic acids, and phospholipids. Phosphite, a reduced form of phosphorus ( $P^{+3}$ ), is found in rivers, lakes, sediments, and oceans, and is of suspected biological origin. However, there is no conclusive evidence of phosphite production in axenic culture, nor any clear mechanistic pathway for phosphite biosynthesis. Given that the phosphate/phosphite redox couple heavily favors oxidation (-690 mV vs. NHE), phosphite has been proposed to be a downstream product in the biosynthesis or degradation of phosphonates, molecules containing a direct phosphorus-carbon bond. This hypothesis forms the basis of my investigations, which primarily aimed to detail biogenic phosphite production in a phosphonate synthesizing organism using an array of optimized analytical techniques for phosphite detection.

I developed an enzymatic, fluorometric assay for phosphite sensing. The method is reliant on the activity of a  $NAD^{+}$ -dependent phosphite dehydrogenase, which through a series of electron transfer reactions, reduces resazurin to the highly fluorescent resorufin. Through the use of a thermostable variant of phosphite dehydrogenase, novel sample preparation methods,

and phosphite standard additions, stable fluorescence readout can be read in 20 minutes with a 3  $\mu$ M limit of detection. The assay's limit of detection and phosphite recovery were assessed in a wide array of biologically- and environmentally-relevant matrices to determine the sensor's robustness. The sensor, along with  $^1\text{H}$ -coupled and  $^1\text{H}$ -decoupled  $^{31}\text{P}$ -NMR, were employed to observe phosphite production in *Streptomyces viridochromogenes*, a gram-positive soil bacterium. Through phosphonate feeding experiments in cell culture and soluble cell lysate, as well as the recombinant expression and purification of CTP-phosphonoformate nucleotidyltransferase (PhpF), phosphite was determined to be the decomposition product of activated phosphonate phosphonoformate-CMP. This work challenged the preexisting assumption that phosphite was a decomposition product of phosphonoformate at biologically-relevant temperatures and pHs. Finally, phosphite was investigated as a mobile, selective P-source for toluene-degrading inoculant *Pseudomonas veronii* in a synthetic soil community (SynCom) as a means of enhancing bioremediation efficiency. A phosphite-oxidizing (*ptxD*<sup>+</sup>) mutant of *Pseudomonas veronii* was constructed that can use phosphite as a sole P source. Soil microcosms with all iterations of the presence or absence of inoculant, SynCom, toluene, and phosphite were propagated, and were monitored for community composition with 16S rRNA gene amplicon sequencing and toluene degradation rates using GC-MS. Phosphite was determined to enhance inoculant abundance most prominently in toluene-free soil microcosm, likely due to the growth substrate being carbon limited.

These investigations provide new insights into the role of phosphite in the biogeochemical P cycle, elucidating both a biological production mechanism of phosphite as well as its bioavailability in soil microbial communities.

## TABLE OF CONTENTS

|      |                                                                                                               |    |
|------|---------------------------------------------------------------------------------------------------------------|----|
| I.   | Phosphite and Life: The Missing Link of the Phosphorus Biogeochemical Cycle                                   |    |
|      | 1                                                                                                             |    |
|      | References.....                                                                                               | 8  |
| II.  | A Fluorometric Assay for High-throughput Phosphite Quantitation in Biological and Environmental Matrices..... | 12 |
|      | Abstract.....                                                                                                 | 12 |
|      | Introduction.....                                                                                             | 13 |
|      | Materials and Methods .....                                                                                   | 15 |
|      | Results and Discussion .....                                                                                  | 23 |
|      | Conclusions.....                                                                                              | 34 |
|      | Acknowledgements.....                                                                                         | 34 |
|      | References.....                                                                                               | 35 |
|      | Supplemental Information .....                                                                                | 38 |
| III. | Phosphite Production by <i>Streptomyces viridochromogenes</i> .....                                           | 41 |
|      | Abstract.....                                                                                                 | 41 |
|      | Introduction.....                                                                                             | 42 |
|      | Materials and Methods .....                                                                                   | 44 |
|      | Results and Discussion .....                                                                                  | 52 |
|      | Conclusion .....                                                                                              | 64 |
|      | Acknowledgements.....                                                                                         | 64 |
|      | References.....                                                                                               | 64 |

|                                                                                                                      |     |
|----------------------------------------------------------------------------------------------------------------------|-----|
| Supplemental Information .....                                                                                       | 67  |
| IV. Phosphite as an Engineered Niche for <i>Pseudomonas veronii</i> in a Synthetic Soil Bacterial<br>Community ..... | 70  |
| Abstract.....                                                                                                        | 70  |
| Introduction.....                                                                                                    | 70  |
| Materials and Methods .....                                                                                          | 73  |
| Results.....                                                                                                         | 82  |
| Discussion.....                                                                                                      | 94  |
| Conclusion .....                                                                                                     | 96  |
| Acknowledgements.....                                                                                                | 96  |
| Data Availability Statement.....                                                                                     | 97  |
| References.....                                                                                                      | 97  |
| Supplemental Information .....                                                                                       | 101 |
| V. Conclusions.....                                                                                                  | 115 |

## LIST OF FIGURES

|                                                                                                                                           |    |
|-------------------------------------------------------------------------------------------------------------------------------------------|----|
| Figure 1. SDS-PAGE gel describing the purification of 17X-PTDH.....                                                                       | 15 |
| Figure 2. SDS-PAGE gel describing the purification of LDH*.....                                                                           | 17 |
| Figure 3. Standard addition fluorescence and linear regression.....                                                                       | 24 |
| Figure 4. The kinetic response of assay fluorescence.....                                                                                 | 25 |
| Figure 5. Phosphite assay kinetic response for 2 µg 17X-PTDH and 100 mM PMS..                                                             | 26 |
| Figure 6. Phosphite assay kinetics profile for varying [PMS].....                                                                         | 27 |
| Figure 7. Standard addition as a means to mitigate matrix effects of the Phi assay....                                                    | 28 |
| Figure 8. Dependence of ionic strength of sample on Phi assay response. ....                                                              | 29 |
| Figure 9. The effect of LDH* treatment on Phi quantitation in <i>E. coli</i> lysate.....                                                  | 30 |
| Figure 10. Box-and-whisker plot of radish wet weight. ....                                                                                | 31 |
| Figure 11. The role of <i>P. stutzeri</i> ( <i>Ps</i> ) in converting Phi to Pi for plant growth in soil.                                 | 32 |
| Figure 12. Oxidation of Phi by <i>P. stutzeri</i> in defined plant tissue media. ....                                                     | 33 |
| Figure 13. SDS-PAGE gel describing the purification of His-SUMO-phpF. ....                                                                | 48 |
| Figure 14. Quantifying His-SUMO-phpF activity by assaying for pyrophosphate.....                                                          | 49 |
| Figure 15. Phi signal in <i>S. viridochromogenes</i> spent medium. ....                                                                   | 52 |
| Figure 16. Phi signal confirmation with Phi- and Pi-spiking. ....                                                                         | 53 |
| Figure 17. Proton-decoupled <sup>31</sup> P-NMR spectrum of <i>S. viridochromogenes</i> spent media<br>sampled throughout incubation..... | 54 |
| Figure 18. Phi signal dependence on PF in <i>S. viridochromogenes</i> spent medium. ....                                                  | 55 |
| Figure 19. Proton-decoupled <sup>31</sup> P-NMR spectra of <i>S. viridochromogenes</i> deletion mutants.                                  | 56 |
| Figure 20. Proton-decoupled <sup>31</sup> P-NMR spectrum of <i>S. viridochromogenes</i> deletion mutant<br>spent media .....              | 57 |



|                                                                                                                                                     |    |
|-----------------------------------------------------------------------------------------------------------------------------------------------------|----|
| Figure 21. Phi production in <i>S. viridochromogenes</i> $\Delta$ phpH lysate.....                                                                  | 58 |
| Figure 22. Proton-decoupled $^{31}\text{P}$ -NMR spectra confirming CTP-phosphonoformate<br>nucleotidyltransferase activity of His-SUMO-phpF.....   | 59 |
| Figure 23. Phi production in phpF enzymatic assays. ....                                                                                            | 61 |
| Figure 24. Proton-decoupled $^{31}\text{P}$ -NMR spectra demonstrating Phi production in trypsin-<br>digested His-SUMO-phpF reaction mixtures. .... | 61 |
| Figure 25. Conversion of PF to Phi by His-SUMO-phpF activity. ....                                                                                  | 62 |
| Figure 26. Preparation of ATMP from <i>S. viridochromogenes</i> spent medium.....                                                                   | 63 |
| Figure 27. Proton-decoupled $^{31}\text{P}$ -NMR spectra of aminotris(methylenephosphonic acid)<br>(ATMP) standard. ....                            | 63 |
| Figure 28. Confirming the insertion and functionality of the <i>ptxD</i> gene in <i>P. veronii</i><br>1YdBTEX2. ....                                | 83 |
| Figure 29. SynCom development in the soil microcosms. ....                                                                                          | 84 |
| Figure 30. Bar plots showing alpha diversity of SynCom in the grow and stable phase.                                                                | 85 |
| Figure 31. Effect of toluene exposure on SynCom.....                                                                                                | 86 |
| Figure 32. Effect of <i>P. veronii</i> <i>ptxD</i> <sup>+</sup> application to toluene-contaminated SynCom.                                         | 87 |
| Figure 33. Phi effect on toluene-remediation system. ....                                                                                           | 89 |
| Figure 34. The Phi effect in liquid SE. ....                                                                                                        | 91 |
| Figure 35. The Phi effect on SynCom development and alkaline phosphatase gene presence<br>in SynCom members.....                                    | 93 |

## LIST OF EQUATIONS

|                                                                        |    |
|------------------------------------------------------------------------|----|
| Equation 1. Error propagation of Phi assay fluorescence readings. .... | 18 |
| Equation 2. Limit of detection for Phi measurement.....                | 19 |
| Equation 3. Error of Phi concentration measurement.....                | 19 |

## LIST OF SCHEMES

|                                                                                                                                                         |    |
|---------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| Scheme 1. Synthesis and degradation pathways for biogenic reduced P. ....                                                                               | 2  |
| Scheme 2. Overview of P chemical industry and the potential for sustainable reduced P products via biogenic Phi.....                                    | 7  |
| Scheme 3. The semi-enzymatic redox cascade coupling Phi oxidation by 17X-PTDH to the ultimate reduction of resazurin to the fluorescent resorufin. .... | 14 |
| Scheme 4. Biosynthesis pathway of Bialaphos, the bioactive element of PTT.....                                                                          | 42 |
| Scheme 5. Proposed mechanism for the formation of Phi by <i>S. viridochromogenes</i> ..                                                                 | 60 |
| Scheme 6. Overview of soil microcosm experimental layout. ....                                                                                          | 73 |

## LIST OF TABLES

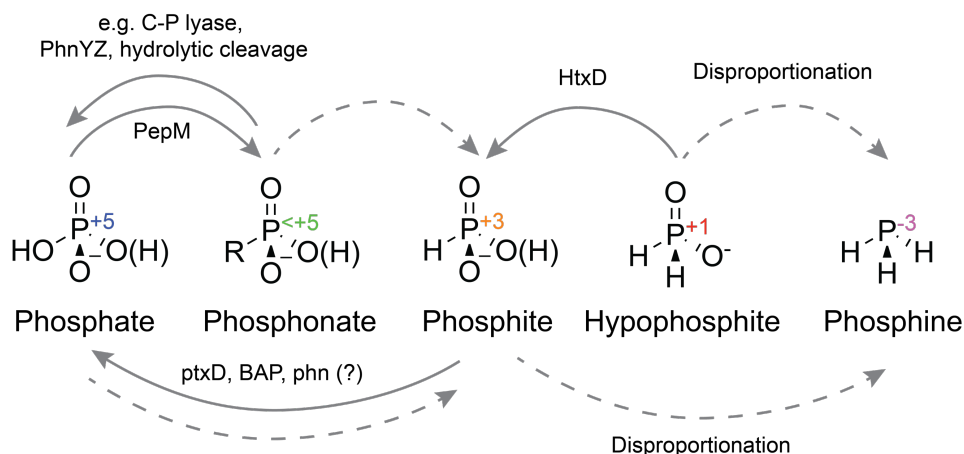
|                                                                                                                     |    |
|---------------------------------------------------------------------------------------------------------------------|----|
| Table 1. Phi assay performance with various biological and environmental matrices spiked with 5 nmol Phi.....       | 28 |
| Table 2. SynCom members.....                                                                                        | 72 |
| Table 3. Primers and DNA sequences used in this study. ....                                                         | 74 |
| Table 4. pBLAST hits for <i>ptxD</i> in SynCom members' annotated genome with E-values < $1 \times 10^{-50}$ . .... | 92 |

# **I. Phosphite and Life: The Missing Link of the Phosphorus Biogeochemical Cycle**

Phosphorus (P) is an essential nutrient for all life that is intimately tied to the global food supply, human health, and microbial productivity. P is found most abundantly in its fully oxidized form (+5): phosphate (Pi,  $\text{H}_2\text{PO}_4^-/\text{HPO}_4^{2-}$ ) and phosphate esters. All canonical P-containing biomolecules, including nucleotides, nucleic acids, phospholipids, and phosphorylated proteins, exist at this oxidation state and are widely considered redox-inert. However, increasing evidence suggests a redox biogeochemical P cycle produces significant reduced (<+5) P pools in some terrestrial and marine environments (**Scheme 1**). Phosphite (Phi,  $\text{HPO}_3^{2-}$ ) is a reduced form (+3) of P that is involved in assimilatory and dissimilatory microbial metabolism and is possibly biogenic. The low reduction potential of the Pi/Phi couple, -690 mV vs. NHE,<sup>1</sup> heavily favors Phi oxidation, however, Phi is kinetically stable and accumulates in the environment. While many abiotic mechanisms of Phi have been proposed and will be discussed here, we primarily will evaluate the evidence for biologically produced Phi. This has implications not only for our understanding of the biogeochemical P cycle and microbial ecology, but for the P chemical industry by offering a potential “green” route to phosphochemical synthesis.

## **Phosphite and Reduced Phosphorus in the Environment**

Phosphite has been observed in modern day lakes, ponds, rivers, sediments, and the ocean.<sup>2-5</sup> Proposed mechanisms for its formation, however, have been largely dependent on the pre-oxygenated conditions of early Earth, including lightning strikes<sup>6</sup>, geothermal pools<sup>7</sup>, and reduction by Fe(II)<sup>8</sup>. These ancient Phi pools explain the early evolution of Phi utilization and the diversity and prevalence of Phi-oxidizing pathways in bacteria.<sup>9,10</sup> Phi concentrations in present day are poorly characterized, due in part to the analytical challenge of rapid, high-throughput Phi detection, as well as the historical assumption that Pi is the only relevant P source for understanding ecosystem productivity. Despite this lack of data, available evidence suggests that reduced P pools can form a significant portion (10-20%) of dissolved P in water samples.<sup>3</sup> Given the oxidizing conditions of present-day Earth render most abiotic mechanisms irrelevant, mechanisms for Phi production are largely hypothesized to be biological in nature.<sup>11,12</sup>



**Scheme 1.** Synthesis and degradation pathways for biogenic reduced P.

The mechanism of biological Phi formation has the potential to “close” the biogeochemical P cycle, which by current understanding is disjointed. While the P cycle was once believed to have no atmospheric component, phosphine ( $\text{PH}_3$ ) was discovered in a

sewage treatment plant and has since been characterized as a trace atmospheric gas that is highly localized in concentration and the result of anaerobic microbial activity.<sup>13–15</sup> Although the mechanism of phosphine production is not clear, it has been proposed to be the product of phosphite and/or hypophosphite disproportionation reactions which form phosphine and  $\text{P}_i$ .<sup>3</sup> It is possible that a similar reaction is mediated biochemically to generate  $\text{P}_i$  for growth, which may outweigh the toxic effects of liberating phosphine. The correlation of phosphine to microbial activity on Earth has led to the proposal of phosphine as a biosignature gas to detect life on other planets, although this hypothesis is controversial.<sup>16,17</sup> It is possible that  $\text{P}_i$  has a biological origin that, under certain thermodynamically favorable conditions, leads to the formation of phosphine and “completes” the biogeochemical P cycle. Regardless, much remains to be discovered about the conditions of phosphine generation, as well as the characterization of biological  $\text{P}_i$  production.

### **Evidence for Biological $\text{P}_i$ Production and Potential Mechanisms**

Direct evidence for biological  $\text{P}_i$  production is scarce, but has been most compellingly demonstrated by van Mooy *et al*, where isotope pulse-trace experiments showed the conversion of radioactively-labeled  $\text{P}_i$  to  $\text{P}_i$  and methylphosphonate (MPn) in seawater enriched with *Trichodesmium* and whole planktonic communities.<sup>4</sup> MPn is the simplest phosphonate (Phn), a reduced P compound with a phosphorus-carbon bond. *Trichodesmium* are both Phn producers and consumers, although these traits are mutually exclusive in almost all cases and vary on a species and strain level.<sup>18,19</sup> Van Mooy *et al.* also found that P(+5) to P(+3) reduction peaked at depths approaching the mesopelagic zone, where archaea compose a substantial fraction (40%) of the total planktonic community. The archaeon *Nitrosopumilus*

*maritimus*, a member of Group I Thaumarchaeota, one of the most abundant organism classes in the mesopelagic zone, is considered one of the primary producers of marine MPn.<sup>20,21</sup> These observations suggest that Phi is associated with the presence of organisms capable of Phn biosynthesis and/or catabolism.

Phn synthesis proceeds in all known cases from phosphoenolpyruvate mutase (PepM), which forms the initial carbon-phosphorus bond from phosphoenolpyruvate (PEP), an intermediate in glycolysis. There are several known oxidative and hydrolytic pathways for Phn degradation,<sup>22-24</sup> but perhaps the most notable pathway due to its implications for global C cycling is the homolytic C-P lyase pathway. Lysis of the C-P bond of MPn and its HMW derivatives by bacterioplankton is considered largely responsible for the emission of methane from marine surface waters.<sup>5</sup> Given the relatively high concentrations of Phn in the marine environment<sup>19</sup>, the association of Phn and Phi would have significant implications for the role of Phi in the global redox P biogeochemical cycle.

Although the mechanism(s) of biological Phi production is unclear, several hypotheses have been put forward. Due to the high energetic barriers for the direct reduction from Pi, Phi production has been previously proposed to be a product of Phn chemistry.<sup>11,12</sup> Phi has been proposed as a product of C-P bond homolysis by the radical S-adenosylmethionine-dependent enzyme PhnJ, where a radical Pi intermediate rearranges to a radical Phi species at  $< +0.1$  V and pH 7.<sup>3,25</sup> However, whether the radical arrangement is relevant to the proposed mechanism of PhnJ<sup>26</sup>, which features a radical species on a thiophosphate intermediate only prior to C-P bond lysis, is unclear and requires further evidence. It has also been proposed based on thermodynamic calculations that Pi can act as an electron sink for NADH in highly reducing, anoxic environments resulting in Phi production, although this is a favorable process

only in environments where sulfate and CO<sub>2</sub> are unavailable as electron acceptors.<sup>27</sup> The only direct evidence suggesting Phi production is a product of Phn biosynthesis is a reported Phi peak in <sup>31</sup>P-NMR from the spent culture media of a  $\Delta phpF$  mutant of Bialaphos-synthesizing bacteria *Streptomyces viridochromogenes*.<sup>28</sup> Although this was attributed to the abiotic decarboxylation of phosphonoformate, the substrate of PhpF, this is an unexpected result given the stability of phosphonoformate at near-neutral pH.<sup>29</sup> Consequently, the link between Phi generation and Phn biosynthesis pathways remains tenuous and further investigation is necessary.

### **Phosphite Oxidizers and the Potential Ecological Role of Phi**

In comparison with biological Phi production pathways, biological Phi oxidation pathways are well-characterized. Phi can be used as an assimilatory P source for bacteria such as *Prochlorococcus*, *Pseudomonas stutzeri*, *Trichodesmium* via the *ptxD* gene coding for the NAD<sup>+</sup>-dependent oxidoreductase phosphite dehydrogenase (PTDH).<sup>30–32</sup> Although not as thoroughly understood, Phi-oxidizing and H<sub>2</sub>-evolving bacterial alkaline phosphatase was identified in *Escherichia coli* and may be applicable to other organisms with promiscuous alkaline phosphatases.<sup>33</sup> The *phn* operon of the C-P lyase pathway, responsible for the cleavage carbon-phosphorus bond of phosphonates, appears to also confer the ability to oxidize Phi through an unknown mechanism, although it cannot sustain the same growth rates as oxidation by *ptxD* and is likely an off-target pathway.<sup>34</sup> Phi can also serve as an adept electron donor in dissimilatory metabolism due to the low redox potential of the Phi/Pi couple, and has been identified in two organisms, *Desulfotignum phosphitoxidans* and *Phosphitispora fastidiosa*, as the sole electron source for the reduction of CO<sub>2</sub>.<sup>35–37</sup>

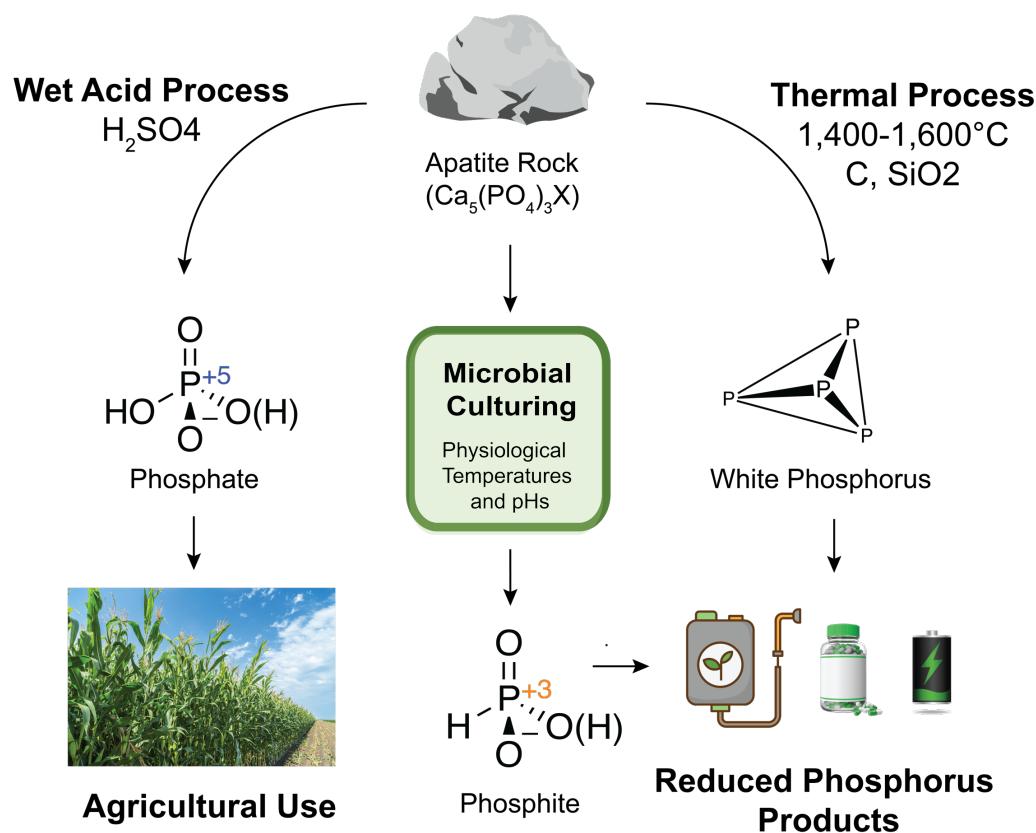
Whether Phi-producing biochemistry should be expected in Phi-oxidizing organisms, or organisms in commensal or symbiotic relationships with Phi-oxidizing organisms, is unclear. Once biological Phi synthetic mechanisms and genetic markers are further elucidated, the ecological impact of Phi production on the surrounding community merits further study. It is possible that in P-limited environments, such as oligotrophic ocean waters, Phi may play a crucial role in resource partitioning for the Phi-producing organism or its symbionts.

### **Applications of Phi Production to the P Industry**

The elucidation of Phi biosynthesis would not only impact our understanding of P biogeochemical cycling, but also provide a novel and sustainable synthetic path to reduced phosphochemicals. The P chemical industry is vital to the global food supply, medical industry, and other essential products, yet current methods of phosphochemical preparation are unsustainable. Phosphorus is obtained for anthropogenic use through the mining of apatite rock (*e.g.*  $\text{Ca}_5(\text{PO}_4)_3\text{F}$ ). Acid treatment of apatite, the “wet process,” consumes 95% of the P supply, producing phosphoric acid, a precursor of phosphate fertilizers (**Scheme 2**).<sup>38</sup> The remaining 5% is treated by the legacy “thermal process,” which involves the reduction of Pi to zerovalent white phosphorus ( $\text{P}_4$ ) at high temperatures (1400°C-1600°C) with coke as the reductant.  $\text{P}_4$  is the feedstock for all man-made phosphochemicals, including pharmaceuticals, herbicides, flame retardants, and battery electrolytes.<sup>39</sup> Due to the high energy costs and toxic reagents involved in the thermal process, there has been increasing interest in alternative synthetic strategies for high-value, reduced P products. An alternative synthetic approach involves the production of Phi, an attractive industrial precursor due to its facile manipulation by Grignard-, Arbuzov- and Michael addition-type reactions. Although Phi is currently



produced by the legacy carbothermal process, previous work has aimed to use mechanochemistry of phosphate anhydrides from yeast cultures, although this still necessitated the use of potent hydride donors and resulted in modest yield.<sup>40</sup> This hybrid biotic-abiotic synthesis approach may be more powerful once Phi production mechanisms have been detailed in axenic cultures. Microbial culturing presents a potentially mild route towards selective Phi synthesis that can operate at moderate temperatures, in aqueous solvent, and near-neutral pH.



**Scheme 2.** Overview of P chemical industry and the potential for sustainable reduced P products via biogenic Phi.

### Exploring the Redox Biogeochemical P Cycle

The following work aims to elucidate the role of Phi in the redox biogeochemical P cycle through several facets. Firstly, the analytical challenge of Phi detection will be addressed through the development of an enzymatic, fluorometric Phi assay that exhibits robust performance in a variety of complex biological and environmental matrices (Chapter II). Equipped with this sensor, a Phi production pathway in *Streptomyces viridochromogenes* is detailed as a product of CTP-phosphonoformate decomposition in its Bialaphos synthetic pathway (Chapter III). Finally, Phi is examined as a selective P source for the proliferation of an inoculant in a model soil community as a means of enhancing bioremediation (Chapter IV).

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## **II. A Fluorometric Assay for High-throughput Phosphite Quantitation in Biological and Environmental Matrices**

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### **Abstract**

Phosphite, the anion of phosphorus acid, is an important metabolite in the global biogeochemical phosphorus cycle and a phosphorus source with unique agricultural properties. As such, methods for detecting phosphite quantitatively and selectively are critical to evidencing phosphorus redox chemistry. Here, we present a fluorescence-based assay for phosphite, utilizing the  $\text{NAD}^+$ -dependent oxidation of phosphite by phosphite dehydrogenase and the subsequent reduction of resazurin to resorufin. With the application of a thermostable phosphite dehydrogenase, a medium-invariant analytical approach, and novel sample preparation methods, the assay is capable of rapid and accurate phosphite quantification with a 3  $\mu\text{M}$  limit of detection in a wide array of biologically- and environmentally-relevant matrices, including bacterial and archaeal cell lysate, seawater, anaerobic digester sludge, and plant tissue. We demonstrate the utility of the assay by quantitating phosphite uptake in a model crop plant in the presence or absence of a phosphite-oxidizing strain of *Pseudomonas stutzeri* as a soil additive, establishing this bacterium as an efficient phosphite converting biofertilizer.

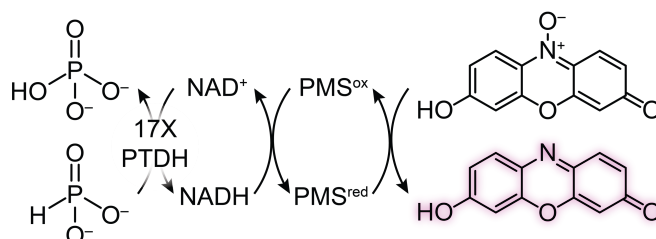
## Introduction

The phosphate ion (Pi), and anhydrides thereof, are known to be central to the biogeochemical cycle of phosphorus, yet less is understood regarding reduced forms of phosphorus, which occupy significant portions of the phosphorus pool, are oxidized biochemically, and are potentially biogenic.<sup>1-8</sup> Phosphite (Phi) is a reduced form of phosphorus that has been observed in lakes, ponds, rivers, oceans, and sediments.<sup>6,9,10</sup> For certain bacterial and archaeal species, Phi can serve as a Pi source by way of the *ptxD* gene coding for phosphite dehydrogenase (PTDH), a NAD<sup>+</sup>-dependent oxidoreductase, which produces Pi necessary for growth in assimilatory metabolism<sup>5</sup> and reducing equivalents (NADH) for biosynthesis in dissimilatory Phi metabolism.<sup>4</sup>

The bacterial metabolism of Phi has inspired biotechnological approaches to phosphorus fertilization using Phi. As a fertilizer, Phi exhibits greater mobility in soil than Pi and inhibits *Phytophthora* root rot.<sup>11,12</sup> Plants cannot naturally oxidize Phi to access Pi, thus Phi must be oxidized by the soil microbiome to generate the Pi necessary for plant growth.<sup>13</sup> Alternatively, transgenic plants encoding the *ptxD* gene facilitate Phi oxidation, supplying the necessary Pi for plant growth upon Phi treatment.<sup>14-16</sup> In the absence of microbiome or an innate mechanism of Phi oxidation, Phi is non-selectively taken up by plants and suppresses the Pi starvation response, which is detrimental to plant growth, and thus Phi can also act as an herbicide under certain conditions.<sup>17,18</sup>

To interrogate the role of Phi in the biogeochemical phosphorus cycle and assess its activity as a fertilizer, analytical tools for Phi quantitation are needed. The primary methods for quantitating reduced forms of phosphorus include ion chromatography (IC), <sup>31</sup>P-nuclear magnetic resonance spectroscopy (<sup>31</sup>P-NMR), and mass spectrometry (MS) which are difficult

to adapt to high-throughput screening and very capital intensive, raising the costs of analysis significantly. Fluorescence-based methods are advantageous due to their sensitivity, ease of analysis, and relatively low cost, but are indirect, and therefore are susceptible to artifacts such as medium effects.<sup>19</sup> An fluorometric method for Phi detection was developed in which the oxidation of Phi and concomitant  $\text{NAD}^+$  reduction by PTDH to the reduction of resazurin to the fluorescent resorufin via the electron carrier phenazine methosulfate (PMS, **Scheme 3**).<sup>20</sup> While this method is cost effective and amenable to high-throughput analysis, it suffers from low and unreliable Phi recoveries in biological samples.



**Scheme 3.** The semi-enzymatic redox cascade coupling Phi oxidation by 17X-PTDH to the ultimate reduction of resazurin to the fluorescent resorufin.

Here, we investigate a series of modifications to the enzyme-coupled fluorescent Phi assay, aiming to improve sensitivity, Phi recovery, and shorten acquisition time. We employed a thermostable variant of PTDH (17X-PTDH)<sup>21</sup> which improved the assay acquisition time by incubating at higher temperatures. The use of a standard addition method increased the reliability of Phi measurements across a range of relevant biological and environmental matrices, including bacterial and archaeal lysate, liquid cell culture media, seawater, anaerobic digester sludge, and plant tissue extract. We also develop a sample preparation procedure for bacterial lysates which enables the use of the assay on matrices with high NADH or NADPH concentrations by treatment with an engineered variant of lactate dehydrogenase (LDH\*) and pyruvate as a hydride acceptor.<sup>22</sup> In a proof-of-principle application, we used this Phi assay to

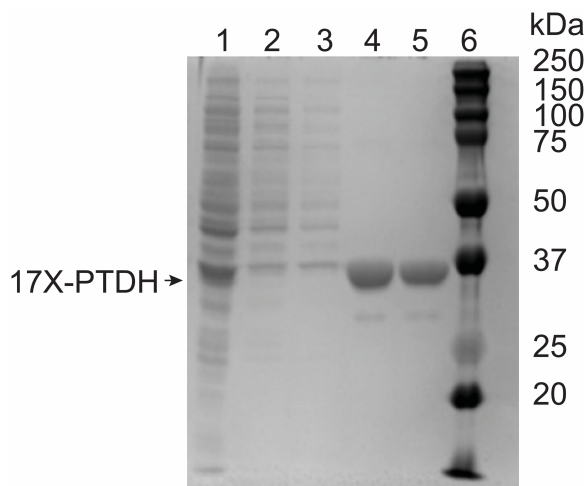


assess Phi distribution in plant tissue when employed as a fertilizer, either alone or in concert with application of *Pseudomonas stutzeri* ATCC 11607, a Phi-oxidizing soil bacterium.<sup>5</sup>

## Materials and Methods

### Materials

LB media (Miller, powder), resazurin, phenazine methosulfate (PMS), 3-(N-morpholino)propanesulfonic acid (MOPS), tris(hydroxymethyl)aminomethane (Tris),  $\beta$ -nicotinamide adenine dinucleotide ( $\text{NAD}^+$ ), phenylmethylsulfonyl fluoride (PMSF), dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), glycerol, imidazole, ampicillin, sodium pyruvate,  $\text{Na}_2\text{HPO}_3 \cdot 5\text{H}_2\text{O}$ , NaCl, KCl,  $\text{CaCl}_2$ ,  $\text{MgCl}_2$ , and  $\text{MgSO}_4$  were purchased from Sigma Aldrich and used without further purification. All solutions were prepared using nanopure ( $>17 \text{ M}\Omega$ ) water.



**Figure 1.** SDS-PAGE gel describing the purification of 17X-PTDH. Lanes contain the *E. coli* BL21(DE3) lysate with overexpressed 17X-PTDH (1), Ni-NTA column flow-through (2, 3), eluted final protein fraction (4,5), and molecular weight ladder (6). The 17X-PTDH is 36 kDa.

### 17X-PTDH Expression and Purification

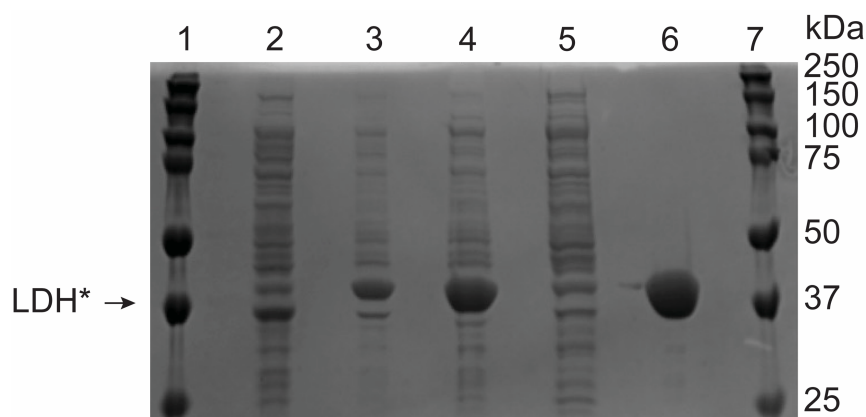
17X-PTDH was expressed and purified from *E. coli* BL21(DE3) cells transformed with a pET-15b vector containing the 17X-PTDH gene using the methods described previously.<sup>21</sup>

The concentration of the purified protein was quantified using absorbance at 280 nm and the previously reported extinction coefficient of  $28,000 \text{ M}^{-1} \text{ cm}^{-1}$ . Protein purity was evaluated by SDS-PAGE (**Figure 1**). The activity of the protein was determined by a previously developed assay monitoring Phi-dependent NADH production by time-dependent absorbance at 340 nm.<sup>21</sup>

### **LDH\* Expression and Purification**

The D176G, I177L, F178W mutant of D-lactate dehydrogenase (LDH\*) from *Lactobacillus delbrueckii* was expressed and purified from *E. coli* BL21(DE3) cells transformed with a pQElac expression vector.<sup>22</sup> Transformants were cultured in 10 mL of LB media overnight with 100  $\mu\text{g/mL}$  ampicillin. Saturated cultures were diluted at a ratio of 1:100 into 1 L of LB media (100  $\mu\text{g/mL}$  ampicillin) and grown to an  $\text{OD}_{600}$  of 0.5, induced with 0.5 mM IPTG, then incubated at 30°C for 24 hrs. The cell pellet containing overexpressed LDH\* was resuspended in Buffer A (20 mM Tris buffer, 1 mM PMSF, 2 mM DTT, 500 mM NaCl, and 10% v/v glycerol at pH 7.6) using 3-5 mL buffer per gram of cell pellet. The cell pellet was homogenized and lysed using a pre-cooled Avestin C3 French Press at 14,000 psi in one pass. The resulting lysate was centrifuged at  $14,636 \times g$  for 10 min and the supernatant was collected. DNA precipitated using 0.2 volume equivalents of 6% w/v streptomycin sulfate. The DNA was separated by centrifugation at  $14,636 \times g$  for 10 min, then the supernatant was collected and centrifuged again for 45 min at  $14,636 \times g$  to remove all remaining insoluble material. The resulting supernatant was loaded onto a Ni-NTA column, pre-equilibrated with Buffer A. The column was washed with 50 column volumes of Buffer B (Buffer A supplemented with 10 mM imidazole). The protein was eluted with approximately 3 column

volumes of Buffer C (Buffer A supplemented with 500 mM imidazole). Fractions containing LDH\* activity were pooled, as determined by NADH oxidation in an assay containing 150 mM sodium phosphate (pH 6.5), 300  $\mu$ M NADH, 2.5 mM  $MgCl_2$ , and 30 mM sodium pyruvate. The resulting pool of LDH\* was concentrated using a 30 kDa Amicon® Ultra-15 centrifugal filter unit (EMD Millipore) at 5,000  $\times$  g for 15 min. The concentrated sample was buffer exchanged with Buffer D (20 mM MOPS, pH 7.6, 100 mM KCl, and 10% v/v glycerol at pH 7.6) using a HiTrap Desalting 5 mL column with Sephadex G-25 resin. The final concentrated protein sample was assessed for purity using SDS-PAGE (**Figure 2**). Activity was determined by adding 1  $\mu$ g of LDH to STET buffer supplemented with 0.5 mM NADH and 30 mM pyruvate. Absorbance at 340 nm was monitored over time at room temperature.



**Figure 2.** SDS-PAGE gel describing the purification of LDH\*. Lanes contain the molecular weight ladder (1), pre-induction *E. coli* BL21(DE3) lysate (2), post-induction *E. coli* BL21(DE3) lysate with overexpressed LDH\* (3), cell lysate (4), Ni-NTA column flow-through (5), final elution protein fraction (6), and the molecular weight ladder (7). The LDH\* is 37 kDa.

### Phosphite Assay

Standard addition samples (sample analytes) were prepared in a master mix for triplicate measurements by diluting 148  $\mu$ L of sample with 17  $\mu$ L of appropriately diluted 1 mM Phi (pH 7.3) or water to a total of 165  $\mu$ L for three 50  $\mu$ L sample analytes. Phi additions typically

spanned 0-5 nmol Phi, but were evaluated up to 15 nmol Phi. When the Phi concentration of the sample is unknown, preference is given towards smaller standard additions to ensure Phi concentrations in the assay reaction mixture remain in the linear range.

The Phi assay reaction mixtures were prepared in a 96-well plate to a total volume of 250  $\mu$ L. Solutions were composed of 50 mM MOPS, 100  $\mu$ M NAD<sup>+</sup>, 100  $\mu$ M (or 150, 200  $\mu$ M) PMS, 100  $\mu$ M resazurin, 1-2  $\mu$ g of 17X-PTDH (100-200 nM final concentration), at pH 7.3, and 50  $\mu$ L of sample analyte, prepared in triplicate. The PMS<sub>ox</sub> intermediate is very light-sensitive and care should be taken to prepare stock solutions and the Phi assay reaction mixture in minimal light. Plates were sealed and incubated and shaken at 37°C for 30 min in a Spark 10M microplate reader (Tecan). After this incubation period, or throughout incubation for the kinetics experiments, fluorescence intensity from resorufin production was excited at 535 nm and measured at 585 nm, with a 20 nm bandpass filter.

### **Data Processing**

The average fluorescence intensity of the samples containing no 17X-PTDH was subtracted from all other fluorescence readings of the assay at each standard addition, and the error was appropriately propagated using Equation 1.

**Equation 1.** Error propagation of Phi assay fluorescence readings.

$$\sigma F = \sqrt{(\sigma a)^2 + (\sigma b)^2}$$

Here  $\sigma F$  is the uncertainty of the fluorescence response to Phi,  $\sigma a$  is the uncertainty of the 17X-PTDH-free samples, and  $\sigma b$  is the uncertainty of total fluorescence response, both defined as the standard deviation of triplicate measurements.

The limit of detection (LOD) was calculated using **Equation 2**.

**Equation 2.** Limit of detection for Phi measurement.

$$LOD = \frac{3 \times \sigma F_0}{m}$$

Here  $m$  is the slope of the standard addition curve and  $\sigma F_0$  is the uncertainty in fluorescence response at 0 nmol of added Phi, calculated using Equation 1. Phi concentrations are obtained by calculating the x-intercept of the linear regression line from the standard additions, dividing by the 50  $\mu$ L sample volume, and accounting for the dilution factor applied in the preparation of the standard additions (150  $\mu$ L used of 165  $\mu$ L prepared). The error of the concentration measurement,  $\sigma C$ , is defined by **Equation 3**, which accounts for the covariance of both slope and intercept.<sup>23</sup>

**Equation 3.** Error of Phi concentration measurement.

$$\sigma C = \frac{\sigma_{xy}}{m} \sqrt{\frac{1}{n} + \frac{\bar{y}^2}{m^2 \sum (x_i - \bar{x})^2}}$$

Here  $\sigma_{xy}$  is the standard deviation of the residuals,  $n$  is the number of data points on the curve,  $x_i$  are the nmol Phi of standards,  $\bar{x}$  is the average concentration of standards,  $\bar{y}$  is the average fluorescence signal of the standards, and  $m$  is the slope of the linear regression line.

### ***Escherichia coli* Sample Preparation**

*E. coli* pellets were resuspended in STET buffer (100 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, and 5% v/v Triton X-100 at pH 8.0) at a ratio of 4 mL buffer/g cell pellet. The pellet was homogenized in buffer and supplied with 1 mM PMSF by a glass homogenizer. Cells were lysed via sonication (5 min processing time with a 1/4" probe, 30 s on-30 s off, at 45% amplitude) and insoluble matter was removed by centrifugation at 14,000  $\times$  g for 10 min. If

relevant, 6 nM of LDH\* and 30 mM sodium pyruvate (pH 7.0) were added to the lysate and shaken at room temperature for 3 min. Protein removal was achieved through passing the lysate through 3 kDa Amicon® Ultra-0.5 centrifugal filter units (EMD Millipore) at 14,000 × g for 30 min. The flow-through was collected for the Phi assay.

### **Ion Chromatography Analysis**

*E. coli* sample standard additions were prepared identically to those used for the Phi assay, and were analyzed by the Laboratory for Environmental Analysis in the University of Georgia Center for Applied Isotope Studies. Samples were run in a DIONEX DX500 chromatography system supplied with an IonPac AS4A column and suppressed conductivity detector. Phi was quantified using the peak area by standard addition.

### **Seawater Sample Preparation**

Seawater was obtained from the Santa Barbara Channel (Santa Barbara, CA, USA) and was passed through a 20 µm filter prior to analysis. Concentrated samples were prepared on a vacuum concentrator.

Artificial seawater for use in the Phi assay consisted of 450 mM NaCl, 10 mM KCl, 9 mM CaCl<sub>2</sub>, 30 mM MgCl<sub>2</sub>, and 16 mM MgSO<sub>4</sub>. To determine the dependence of ionic strength on Phi response, individual components of artificial seawater recipe were removed or doubled in concentration and applied to the Phi assay.

### **Anaerobic Digester Sample Preparation**

Anaerobic digester sludge was collected from the El Estero Water Resource Center (Santa Barbara, CA, USA) using 1 L plastic bottles, which were immediately placed in an anaerobic chamber (Coy Laboratory Products) for incubation at 37°C. When needed, aliquots were removed from the anaerobic chamber and filtered using a 0.22 µm syringe filter (VWR) before use in the Phi assay.

### **Archaeal Lysate Preparation**

*Nitrosopumilus maritimus* SCM1 was grown in Synthetic Crenarchaeota Medium (SCM) as described previously.<sup>24</sup> Cultures were grown at 30°C in the dark with no shaking, and growth was monitored by measuring NO<sub>2</sub><sup>-</sup> production with Griess reagent.<sup>25</sup> Once cultures reached early stationary phase, cells were concentrated 1000-fold using tangential flow filtration through a 100 kDa Vivaflow™ 200 Crossflow Cassette (Sartorius). The resulting concentrated cells were lysed using three cycles of freezing in liquid nitrogen and thawing in a 37°C water bath.

### **Radish Growth**

Radish seed preparation was adapted from Liu and colleagues.<sup>26</sup> Seeds (Cherry Giant, Burpee) were sterilized using 30% bleach for 15 minutes at ~200 seeds per 10 mL. The seeds were then washed 3 times with sterile 50 mM NaCl. The seeds were sorted in a sterile 96-well plate and 250 µL sterile 50 mM NaCl or *P. stutzeri* ATCC 11607 (grown in LB broth to an OD<sub>600</sub> of ~0.25) was added. The plates were incubated at 30°C for 24 hrs.

Potting soil was sterilized by autoclaving for 1 hr and placed in an ethanol-sterilized polypropylene planter divided into four 30 cm × 30 cm × 25 cm quadrants. Radish seeds were

planted ~2 cm deep with 2-3 seeds planted in every 10 cm × 10 cm area and placed in a growth chamber (Percival) at a light intensity of 220  $\mu\text{mol photons/m}^2\text{s}$ . Radishes grown on Phi with sterile soil and those grown on Phi with *P. stutzeri* treatment were watered daily with 15 mM sodium phosphite (pH 7). Radishes grown in the control planter and those grown with only *P. stutzeri* treatment were watered daily with DI H<sub>2</sub>O. After one week, sprouts were thinned so radishes were at least 5 cm apart. Weekly, beginning 7 days after planting seeds, *P. stutzeri* cultures were applied to the soil of the relevant quadrants. Single colonies of *P. stutzeri* were picked to inoculate overnight cultures. Overnight cultures were diluted in LB liquid media at a 1:50 dilution and grown at 37°C until an OD<sub>600</sub> of 0.6-0.8 was reached. Approximately 50 mL of culture was then applied per 10 cm × 10 cm area of soil.

Radishes were also grown in Murashige and Skoog (MS) medium. This defined medium was prepared with varying phosphorus sources: 1.25 mM Pi, 1.25 mM Phi, or no P source. In addition, 10 mg/L natamycin was supplemented to the media to prevent fungal contamination. Seeds were sterilized and incubated in either sterile 50 mM NaCl or in *P. stutzeri* culture. *P. stutzeri* cultures used for seed growth on MS medium were grown to OD<sub>600</sub> of ~0.25 in MOPS minimal medium<sup>27</sup> with Phi as the sole P source, and were then washed 3 × with sterile MOPS minimal media containing no P source. After 24 hours, seeds were placed in MS media culture boxes in sterile conditions. Seeds were kept in the dark at 23°C until germination occurred, then were subjected to the previously described diurnal light treatment. Sprouted seeds were harvested after 15 days.

### **Radish Leaf and Root Lysis**



Radishes were sourced either from the previously discussed laboratory growth, or commercially for Phi recovery and LOD determination. Leaves and roots were separated, cleaned with DI water, patted dry, and weighed. Seedlings grown on MS media were pooled with 3-5 seedlings per single Phi extraction. The plant material was then frozen in a mortar cooled with liquid nitrogen and ground with a pestle until a fine powder was obtained. The powder was then resuspended in STET buffer with 1 mM PMSF at a ratio of 4 mL STET/g plant wet weight. The suspension was centrifuged at  $14,000 \times g$  for 10 minutes. The resulting supernatant was passed through a 3 kDa Amicon® Ultra-0.5 centrifugal filter unit (EMD Millipore) at  $14,000 \times g$  for 30 min. The flow-through was collected for analysis in the Phi assay. To measure Phi concentrations within the range of the Phi assay, the lysate was diluted at a 1:100 ratio with STET buffer.

## **Results and Discussion**

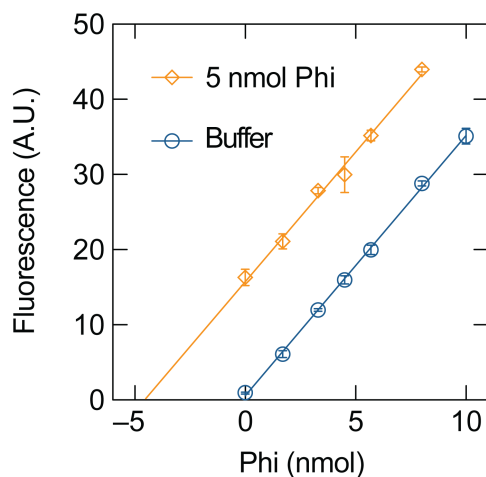
### **Standard Addition Method**

Medium effects can have substantial impact on enzyme-based and fluorescence-based assays, and must be accounted for to ensure accurate and precise analyte measurements. The standard addition method, in principle, accounts for such medium effects, including enzyme inhibition, off-pathway chemical processes, and fluorophore quantum yield variance.<sup>23</sup> We have thus investigated the standard addition method in a PTDH-coupled fluorescence-based assay for Phi detection to develop a medium invariant assay for Phi across several biologically relevant milieu.

In the Phi standard addition assay employed herein standard addition curves are generated for a sample analyte by sequential standard additions of Phi (**Figure 3**), and measuring the differential fluorescence signal from analyte treated with, and without, added 17X-PTDH at

each Phi addition. The responsiveness of the method is measured by the slope, accounting for enzyme inhibition and fluorescent quantum yield effects, and the subtraction of the response without 17X-PTDH treatment accounts for non-specific resazurin reduction. Using this method, we determined the Phi quantitation for a 5 nanomole (nmol) spike, yielding a measured total Phi of  $5.0 \pm 0.4$  nmol, corresponding to a concentration of  $100 \pm 10$   $\mu$ M.

From the 5 nmol spike sample the LOD for the standard addition method in assay buffer was determined to be  $0.15 \pm 0.02$  nmol, corresponding to  $\geq 3.4$   $\mu$ M Phi in the original sample. This LOD reported is a nearly two-fold improvement from the previously reported value for the analogous Phi detection assay. The LOD is also competitive with alternative methods developed for Phi detection, including  $^{31}\text{P}$ -NMR (630-1000  $\mu$ M LOD)<sup>28</sup> or ion chromatography (0.04-0.39  $\mu$ M LOD),<sup>29,30</sup> but with the advantage of the relative ease of analysis, quicker processing time, and lower cost.



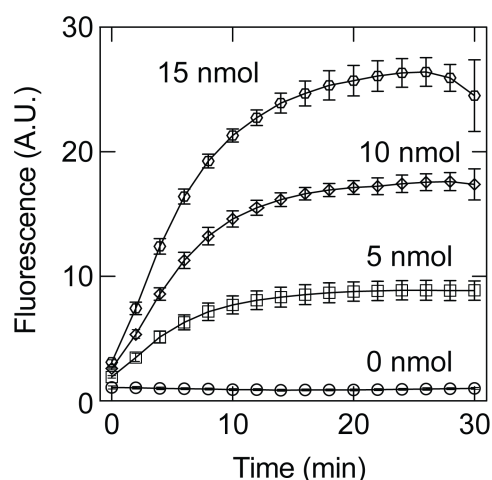
**Figure 3.** Standard addition fluorescence and linear regression. The Phi assay was applied to assay buffer (blue circles) and assay buffer supplemented with 5 nmol Phi (orange diamonds). The dilution-adjusted Phi concentration was determined to be  $5.0 \pm 0.4$  nmol ( $100 \pm 10$   $\mu$ M) from the x-intercept.

### Optimization of Phi Assay Kinetics

To optimize the kinetics of the assay we investigated the redox cascade in the Phi assay system (**Scheme 3**). The recombinantly produced 17X-PTDH, responsible for the selective

and rapid oxidation of Phi, exhibited a  $k_{\text{cat}}$  of  $3 \text{ s}^{-1}$  at  $25^{\circ}\text{C}$ , in agreement with previously reported values,<sup>21</sup> and an increase to  $7 \text{ s}^{-1}$  at  $37^{\circ}\text{C}$ . With no further modification to the assay, the resorufin response was stable within 30 minutes of reaction time with 100 nM 17X-PTDH, half the time of the originally reported assay (**Figure 4**).<sup>20</sup> The reaction time could be further shortened by doubling the concentration of 17X-PTDH (200 nM), cutting the reaction time to as little as 15 minutes and increasing the Phi response (**Figure 5**). The increase in response is likely due to a lower relative population of inhibited PTDH, kinetically privileging the desired chemistry over side reactions, or both.

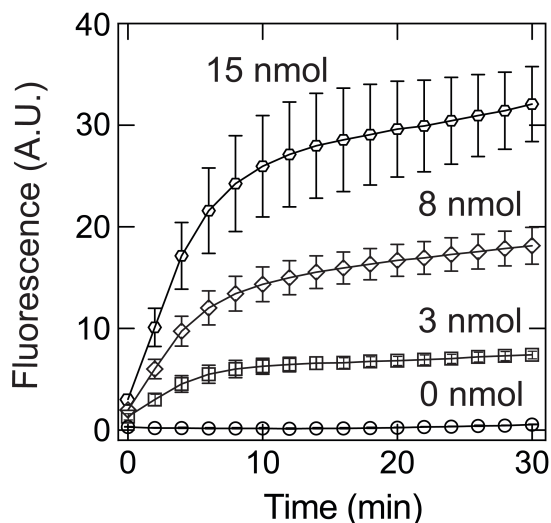
Two additional parameters may affect the kinetic response of the assay within the coupled



**Figure 4.** The kinetic response of assay fluorescence. Phi assay mixtures containing 0 (circles), 5 (squares), 10 (diamonds), and 15 nmol (hexagons) Phi were incubated for 30 minutes at  $37^{\circ}\text{C}$  with shaking, and fluorescence measurements were taken every 2 minutes.

assay, resazurin and PMS. Resazurin has a limited window of optimization due to the inner-filter effect, which lowers fluorescence responses at high concentration.<sup>20</sup> We thus sought to investigate the effect of the mediator PMS on the kinetic response of the coupled assay. PMS is an essential electron transfer agent of the assay reaction, without which no Phi-dependent resorufin production is observed (data not shown). Increasing [PMS] from  $100 \mu\text{M}$  to  $200 \mu\text{M}$

showed minimal effect on the kinetic profiles and the LOD, although it moderately increased Phi response (**Figure 6**). This result is consistent with our prior observation that 17X-PTDH appears to be rate determining in the reaction kinetics (**Figure 5**).



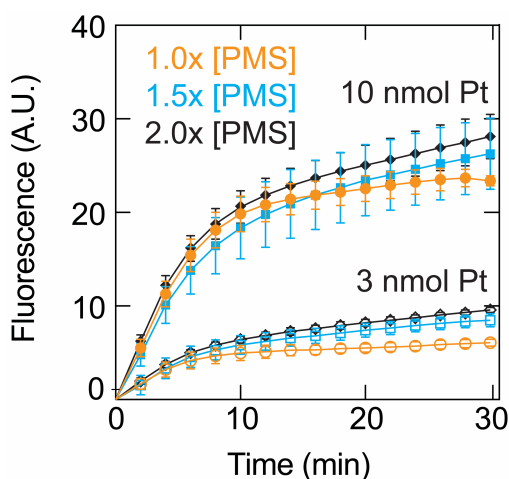
**Figure 5.** Phosphite assay kinetic response for 2  $\mu$ g 17X-PTDH and 100 mM PMS. Assay reactions containing 0 (circles), 3 (squares), 8 (diamonds), and 15 (hexagons) nmol Phi are shown.

### Matrix Effects on Phi Recovery and LOD

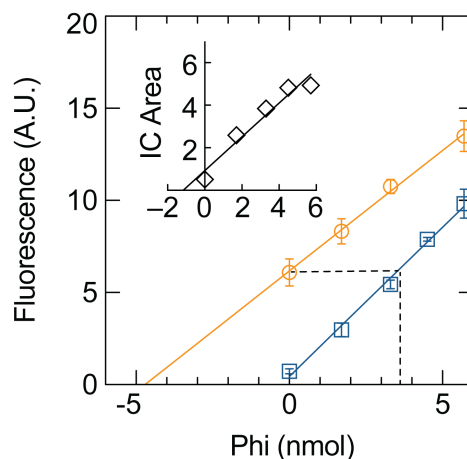
The response of the Phi assay is highly dependent on the sample matrix. This is evident when comparing the standard addition method to an external calibration curve for a complex matrix, such as *E. coli* lysate (**Figure 7**). The standard addition protocol yields a lower slope response when measured in cell lysate than in buffer, resulting in increased error in the extrapolated quantitation. The advantage of the standard addition approach is readily understood from the Phi quantitation and recovery, the standard addition method yielded  $104 \pm 6\%$  recovery of a 5 nmol Phi spike whereas external calibration yielded only  $78 \pm 7\%$  (**Figure 7**, dashed lines). The results were externally validated by standard addition ion

chromatography, which confirmed the Phi specificity, yet yielded lower recovery rates ( $25 \pm 13\%$ , **Figure 7** inset), further supporting the fluorescence methodology.

The reduced Phi response in *E. coli* lysate relative to buffer solution implies that the LOD is matrix dependent and should be reevaluated depending on the sample matrix involved. We investigated several relevant biological and environmental sample matrices to assess Phi recovery and LOD (**Table 1**). Sample matrices were chosen based on compelling environmental observations of putatively biogenic reduced phosphorus compounds, including in seawater and anaerobic digesters.<sup>1,31,32</sup> We also investigated plant tissue lysate, where Phi transport may have agricultural interest.<sup>14,17</sup> Most matrices show high Phi recovery (94-105%) and LODs in the range of 3 to 21  $\mu\text{M}$  (0.15 to 0.96 nmol). Anaerobic digester recoveries were low (77%), likely due to the dynamic chemical and biological composition of digester fluid.



**Figure 6.** Phosphite assay kinetics profile for varying [PMS]. Assay conditions include 2  $\mu\text{g}$  17X-PTDH and 100 (orange circles), 150 (blue squares), or 200 mM (black diamonds) PMS. Assay reactions containing 3 nmol (open shapes) and 10 nmol (filled shapes) Phi are shown.



**Figure 7.** Standard addition as a means to mitigate matrix effects of the Phi assay. Phi recovery in 5 nmol Phi-spiked *E. coli* lysate using the standard addition (orange circles) versus an external calibration in assay buffer (blue squares). Extrapolation of the 0 Phi added to the external calibration shown in dashed lines. Phi recovery by ion chromatography (IC) is shown in the inset (black diamonds).

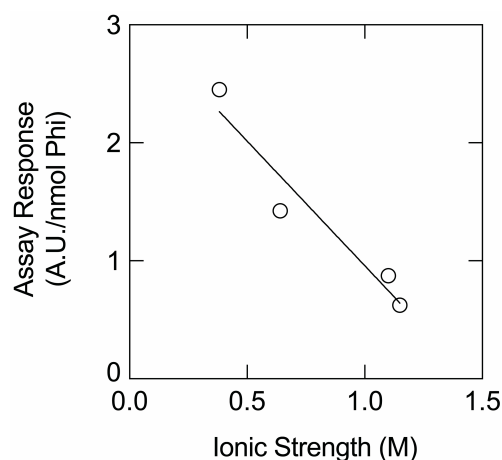
To identify the matrix elements that affect the LOD performance of the assay we initially examined the effect of salt ions within the composition of seawater. We prepared a series of artificial seawater matrices in which one salt was omitted to examine the contribution of individual ions on the Phi assay. Although no salt was particularly inhibiting (data not shown),

**Table 1.** Phi assay performance with various biological and environmental matrices spiked with 5 nmol Phi.

| Sample Matrix              | Recovery (%) | LOD ( $\mu$ M)   |
|----------------------------|--------------|------------------|
| Water                      | 101 $\pm$ 8  | 3.4 $\pm$ 0.3    |
| <i>E. coli</i> lysate      | 104 $\pm$ 6  | 6.8 – 45 $\pm$ 3 |
| Seawater                   | 101 $\pm$ 12 | 13 $\pm$ 9       |
| 2 $\times$ Seawater        | 103 $\pm$ 16 | 21 $\pm$ 9       |
| Radish leaf lysate         | 105 $\pm$ 14 | 13 $\pm$ 1       |
| Radish root lysate         | 94 $\pm$ 9   | 13 $\pm$ 8       |
| Anaerobic digester         | 77 $\pm$ 5   | 12 $\pm$ 3       |
| <i>N. maritimus</i> lysate | N.D.         | 14 $\pm$ 4       |

N.D. not determined

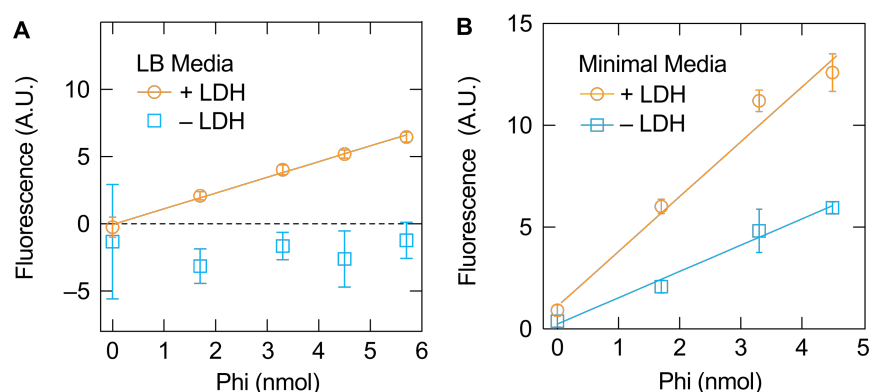
a negative linear relationship between overall ionic strength and Phi response in the assay was found (**Figure 8**). Thus, those interested in applying this assay to seawater concentrated further than 50% its original volume should consider preparatory steps for salt removal, such as ion exchange or electrolysis.<sup>33,34</sup>



**Figure 8.** Dependence of ionic strength of sample on Phi assay response. Assay response is defined as the slope of the standard addition curve in fluorescence (A.U.) versus nmol Phi added.

Another sample matrix element that may negatively affect the Phi assay performance when applied to cellular lysate are remnant NAD(P)H pools, which would reduce resazurin, short-circuiting the Phi-dependent NAD(P)H production by PTDH, and lowering the assay response. NADH is also a product of the PTDH enzyme and a potent competitive inhibitor with  $K_i$  values of  $115 \pm 6 \mu\text{M}$  and  $233 \pm 15 \mu\text{M}$  for Phi and  $\text{NAD}^+$ , respectively.<sup>35</sup> To exhaust metabolic NAD(P)H pools from cell lysate we explored an enzymatic treatment, using a variant of lactate dehydrogenase (D176G, I177L, F178W, termed LDH\* herein) from *Lactobacillus delbrueckii* with high activities for both NADH and NADPH as substrates for the reduction of pyruvate.<sup>22</sup> Using this enzyme, pre-incubation of biological samples with LDH\* and excess pyruvate should rapidly oxidize NAD(P)H, forming  $\text{NAD(P)}^+$  and lactate. This LDH\* was recombinantly expressed, purified, and shown to be active, with a  $k_{\text{cat}}$  of  $1,330 \pm 30 \text{ s}^{-1}$  (**Supplemental Information Figure 1.1**).

The workflow for preparing *E. coli*, and all other cells, for the Phi assay is described in the Materials and Methods and shown schematically in **Supplemental Information Figure 1.2**. Upon addition of LDH\* and pyruvate to lysed cells, consumption of NAD(P)H can be measured by observing the absorbance change at 340 nm (**Supplemental Figure 1.3**). The LDH\* treatment can be either essential for functional response or just beneficial to the Phi assay performance depending on the medium. *E. coli* lysate grown in LB requires LDH treatment to show any Phi response (**Figure 9A**, LOD of 45  $\mu\text{M}$ ), whereas *E. coli* grown in minimal MOPS media show improvement in Phi response (**Figure 9B**). In the minimal media matrix, the LOD was improved from 15  $\mu\text{M}$  to 6.8  $\mu\text{M}$  Phi with LDH\* treatment. Cytosolic NAD(P)H/NAD(P)<sup>+</sup> levels may vary slightly depending on many growth parameters including media, aeration, and cell cycle, and thus treatment with LDH can mitigate these variations and maintain assay performance. With the application of the LDH\* treatment, a LOD for *E. coli* lysate of 0.31 nmol can be achieved. This is equivalent to an intracellular concentration of 24  $\mu\text{M}$ , assuming a cell volume 1  $\mu\text{m}^3$ . This intracellular LOD is similar to the metabolites of low concentration in *E. coli* (e.g. 3',5'-cyclic adenosine monophosphate,



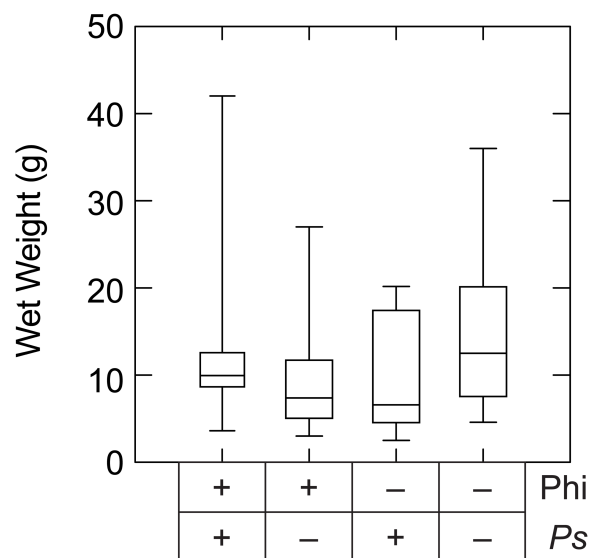
**Figure 9.** The effect of LDH\* treatment on Phi quantitation in *E. coli* lysate. **A** Standard addition curve from *E. coli* grown in LB media and **B** minimal MOPS media with (orange circles) and without (blue squares) LDH\* treatment.



cAMP),<sup>36</sup> making this Phi assay potentially suitable for determining Phi concentrations in cells at even trace levels.

### ***P. stutzeri* ATCC 11607 as a Biofertilizer**

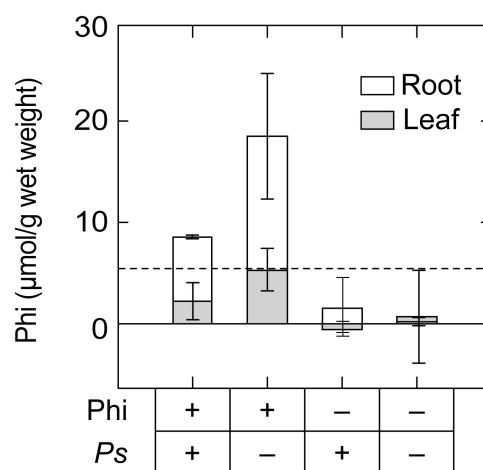
To demonstrate the utility of the standard addition Phi assay in biochemically relevant and challenging matrixes we sought to track Phi distribution in plant-soil microbiome symbionts. Phi has been investigated as an alternative phosphorus source, yet requires oxidation by the soil microbiome to produce the necessary Pi and to maintain the fidelity of the Pi starvation response.



**Figure 10.** Box-and-whisker plot of radish wet weight. Radishes were grown in soil containing Phi, *P. stutzeri* (*Ps*), both or neither.

We selected the naturally soil-dwelling and Phi oxidizing *P. stutzeri* ATCC 11607 as a proof-of-concept candidate for a soil microbiome symbiont with radishes (*Raphanus raphanistrum*) as a test plant. Radishes were grown in autoclave-sterilized soil and tested for their response to both Phi as a phosphorus source and *P. stutzeri* as a Phi oxidizing agent. Both the application of *P. stutzeri* or Phi alone yielded small drops in plant biomass relative

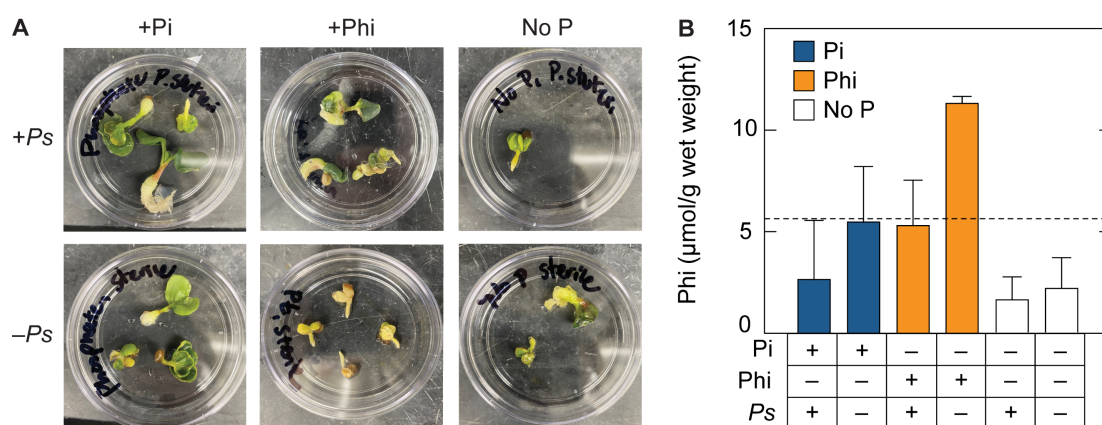
to the sterile, no Phi control, whereas the application of both *P. stutzeri* and Phi recovered total plant biomass (**Figure 10**). These data suggest that both Phi and *P. stutzeri* may be slightly inhibitory to plant growth alone, but in concert, convert Phi to Pi, making phosphorus bioavailable and non-toxic. The endogenous Pi in the commercially available soil provides sufficient phosphorus to maintain growth in all conditions. The action of *P. stutzeri* in oxidizing Phi was confirmed by the application of the Phi assay to the radish root and leaf lysate, which revealed Phi oxidation by *P. stutzeri*, via the drop in Phi concentration in the root biomass (**Figure 11**). Interestingly, it is clear that the Phi distribution is largely concentrated in the root, and the assay developed herein may be useful for tracking Phi dynamics spatio-temporally.



**Figure 11.** The role of *P. stutzeri* (*Ps*) in converting Phi to Pi for plant growth in soil. Phi distribution with the root or leaf biomass in the presence (+) or absence (-) of applied Phi or *Ps*. The LOD for plant tissue (13  $\mu$ M) is marked by the dashed line.

The autoclaved soil contains endogenous bioavailable Pi, potentially limiting the metabolic need for *P. stutzeri* or the plant to rely on Phi oxidation. To increase the nutritional pressure towards Phi oxidation, we investigated Phi oxidation by *P. stutzeri* on defined MS media for growth, rather than sterile soil, either containing Phi, Pi, or a negative control with

no phosphorus supplied. Both sterile and *P. stutzeri*-treated seeds displayed healthy growth on Pi as the phosphorus source (**Figure 12A**, left column), suggesting *P. stutzeri* has minimal impact on plant growth in these conditions. Phi toxicity under sterile conditions was evident due to the apparent yellowing and poor growth shown by these seedlings (**Figure 12A**, bottom center).<sup>37</sup> Seeds treated with *P. stutzeri* ATCC 11607, however, exhibited no signs of Phi-toxicity and grew at a rate closer to, but slower than, the seedlings grown on Pi (**Figure 12A**, top center). When no phosphorus source was supplied, seedlings with and without *P. stutzeri* application exhibited large brown spots on the leaves, stunted growth, and low germination rates (**Figure 12A**, right column). The observation that *P. stutzeri* enhances radish growth on Phi media, but does not in the absence of a phosphorus source, suggests that the effect of *P. stutzeri* ATCC 11607 on plant growth is linked to its ability to oxidize Phi. To confirm that Phi-oxidation was occurring and alleviating Phi uptake by the plant, seedling Phi content was assessed using the Phi assay. Phi concentrations were only above the 13  $\mu\text{M}$  limit of detection in the case of sterile seeds grown on Phi, which yielded  $28.6 \pm 0.8 \mu\text{M}$  of Phi in the assay, well above the LOD of 13  $\mu\text{M}$ , corresponding to 11  $\mu\text{mol}$  Pt/g radish wet weight (**Figure**



**Figure 12.** Oxidation of Phi by *P. stutzeri* in defined plant tissue media. **A** Radish seedlings harvested after 15 days of growth with supplemented Pi, Phi, or no phosphorus (P) source in the presence and absence of applied *P. stutzeri*. **B** Observed Phi concentrations in radish tissues. The LOD for plant tissue is marked by the dashed line.

**12B).** This suggests that *P. stutzeri* ATCC 11607 can eliminate Phi-toxicity by the conversion of Phi to Pi, which in turn supplies Pi to the plant. Collectively, the results support a role of *P. stutzeri* as a soil or media supported symbiont for the effective use of Phi as a phosphorus source in fertilization.

## **Conclusions**

Phosphite is increasingly recognized as a biogeochemically relevant phosphorus species as well as a potential fertilizer with unique properties. Here we describe a low-cost, high-throughput Phi detection method that enables micromolar-level detection in a variety of complex matrices with minimal sample preparation and fluorescence readout in 15 minutes. We leveraged this assay to assess *P. stutzeri* as a Phi-oxidizing symbiont for radishes as a model crop plant. The versatility of this enzyme-coupled assay will enable discovery in phosphorus redox chemistry in diverse environments where redox cycling may significantly contribute to the phosphorus and other elemental biogeochemical cycles.

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## Supplemental Information

### LIST OF SUPPLEMENTAL FIGURES

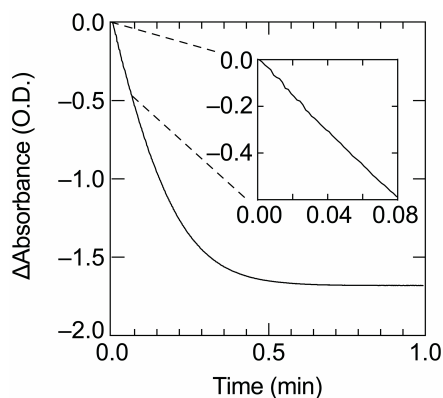
Supplemental Figure 1. 1. Pyruvate-dependent NADH oxidation activity of purified LDH\*.

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Supplemental Figure 1. 3. Schematic representation of sample preparation for the Phi assay.

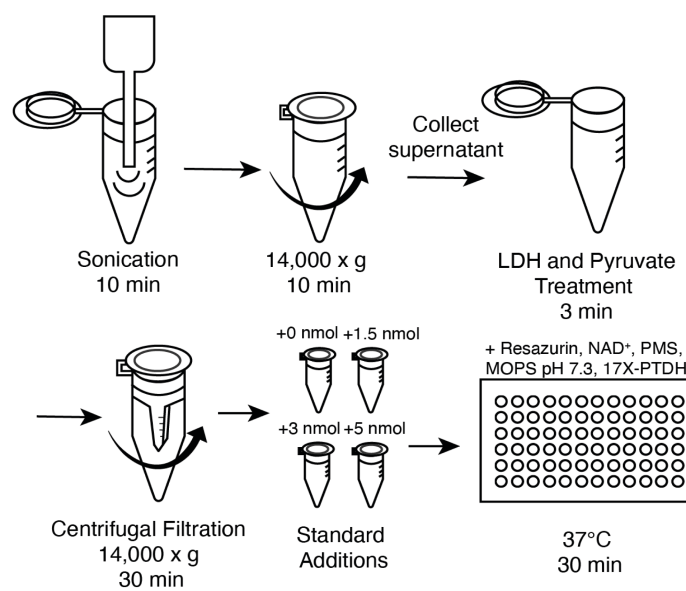
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Supplemental Figure 1. 2. Effect of LDH\* and pyruvate treatment on *E. coli* cell lysate. 40

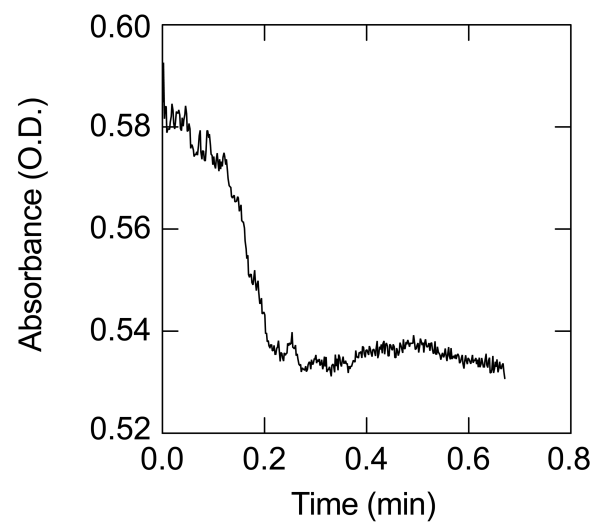


**Supplemental Figure 1. 1.** Pyruvate-dependent NADH oxidation activity of purified LDH\*. Assay takes place in STET buffer and activity is determined by the change in absorbance at 340 nm. The  $k_{\text{cat}}$  was determined from the pseudo-first order regime within the first 5 seconds to be  $1,330 \pm 30 \text{ s}^{-1}$ .





**Supplemental Figure 1. 2.** Schematic representation of sample preparation for the Phi assay.



**Supplemental Figure 1. 3.** Effect of LDH\* and pyruvate treatment on *E. coli* cell lysate. Absorbance of NAD(P)H is measured at 340 nm.

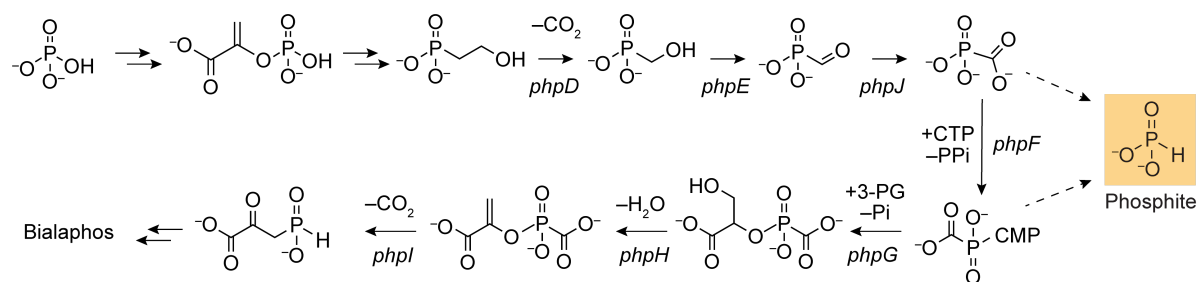
### III. Phosphite Production by *Streptomyces viridochromogenes*

#### Abstract

The industrial production of phosphochemicals is an energy intensive process that involves the reduction of the phosphate mineral apatite to white phosphorus, a toxic, reactive, and environmentally-harmful intermediate. Phosphite is an attractive and activated form of phosphorus that is readily integrated into conventional synthetic processes to phosphochemicals and represents an alternative “green” route to phosphochemical synthesis that bypasses the legacy carbothermal method of white phosphorus synthesis. The biochemical reduction of phosphate to phosphite has been implicated from environmental and microbial studies, yet the biological reduction of phosphate to phosphite has never been definitively established in axenic culture. Here we characterize phosphite production by *Streptomyces viridochromogenes* and demonstrate that phosphite is an abiological product of CTP-phosphonoformate decomposition, an intermediate step in the biosynthesis of the antibiotic phosphinothricin tripeptide (PTT). The product of this enzymatic reaction, phosphonoformyl-CMP (PF-CMP), yields an “activated” phosphonoformate for decarboxylation and hydrolysis at biological temperatures and pHs. Using *S. viridochromogenes* spent media, we demonstrate a hybrid biotic-abiotic synthesis of the commodity metal chelator aminotris(methylenephosphonic acid) (ATMP), demonstrating the potential for hybrid biotechnological synthesis of phosphochemicals.

## Introduction

The phosphorus (P) industry lacks sustainable methods to produce phosphochemicals used in a wide range of applications, including commodity chemicals, pharmaceuticals, herbicides, flame retardants, and battery electrolytes.<sup>1,2</sup> The legacy “carbothermal” process for activating P for phosphochemical synthesis reduces phosphorous acid ( $\text{H}_3\text{PO}_4$ ,  $\text{P}_i$ ), derived from apatite rock, to white phosphorus ( $\text{P}_4$ ) using high temperatures ( $>1000^\circ\text{C}$ ) and fossil fuels as the reductant.<sup>3</sup> This  $\text{P}_4$  product can then generate a variety of reactive intermediates via



**Scheme 4.** Biosynthesis pathway of Bialaphos, the bioactive element of PTT.

exothermal transformations such as disproportionation to generate phosphine ( $\text{PH}_3$ ) and hypophosphorous acid ( $\text{H}_2\text{PO}_2$ ), reactions with oxygen to form various phosphorus oxides, or reactions with halogens to form the versatile phosphochemical precursors. This phosphochemical cycle is energy-intensive, inefficient, and produces a variety of reactive and toxic intermediates.

An alternative route to diverse phosphochemicals is the direct reduction of  $\text{P}_i$  to phosphite (Phi). Phi can undergo facile manipulation through Grignard-, Arbuzov- and Michael addition-type reactions to yield phosphonates and can disproportionate to generate  $\text{PH}_3$  at moderate temperatures.<sup>2</sup> Currently, Phi is produced by the carbothermal process through the oxidation of  $\text{P}_4$  under  $\text{O}_2$ -limited conditions or the hydrolysis of  $\text{PCl}_3$ . This circuitous synthetic method is employed due to the lack of viable chemical approaches to the direct two-electron

reduction of  $P_i$  to  $\Phi$ . The direct reduction of  $P_i$  is thermodynamically challenging; the reduction potential of  $P_i$  reduction to  $\Phi$  is 280 mV more negative than proton reduction in water.<sup>4</sup> Mechanochemistry of phosphate anhydrides, using potent hydride donors such as potassium hydride, results in  $\Phi$  production, yet this process produces a complex product mixture and modest phosphite yields.<sup>5</sup> Thus, developing alternative methods of  $\Phi$  production that are selective and operate at moderate temperatures in aqueous solution is an unsolved challenge.<sup>6</sup>

There is abundant evidence of environmental accumulation of reduced P species, including  $\Phi$ , that appears to be of biogenic origin.<sup>7–12</sup> Microbial reduction of  $P_i$  to  $\Phi$  could offer a sustainable route to  $\Phi$  production. The microbial production of phosphonates ( $RPO_3H_2$ ) is well-documented from phosphoenol pyruvate via the enzyme PEP mutase (PepM).<sup>13</sup> Phosphonates are an attractive “activated” P precursor in a potential microbial  $P_i$  reduction pathway. However, the conversion of phosphonates to  $\Phi$  has never been conclusively demonstrated in axenic cultures, nor have the biochemical mechanisms been fully elucidated.

In this study, we report the  $\Phi$ -producing activity of *Streptomyces viridochromogenes* DSM 40736 in the biosynthetic pathway of the broad-spectrum herbicide Bialaphos (**Scheme 4**). Previous work detailing the Bialaphos biosynthetic pathway proposed  $\Phi$  as a decarboxylation product of the intermediate phosphonoformate (PF), yet this proposal was inconsistent with the known stability of PF towards decarboxylation at near neutral pH.<sup>14–16</sup> Through PF feeding experiments and the expression and purification of recombinant CTP-phosphonoformate nucleotidyltransferase (phpF), we show that  $\Phi$  is produced at biologically relevant pHs and temperatures via a phosphonoformylated CMP intermediate. Finally, we evaluate PF as potential feedstocks for sustainable reduced P production by using spent culture

medium for the synthesis of the metal-chelating agent aminotris(methylenephosphonic acid) (ATMP).

## **Materials and Methods**

### **Materials**

Sodium phosphonoformate tribasic pentahydrate (PF), NaCl, KCl, 3-(N-morpholino)propanesulfonic acid (MOPS), 2-(N-morpholino)ethanesulfonic acid (MES), aminotris(methylenephosphonic acid) ATMP, cytidine 5'-triphosphate disodium salt (CTP), adenosine 5'-triphosphate disodium salt (ATP), 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt (TSP), NH<sub>4</sub>Cl, phenazine methosulfate (PMS),  $\beta$ -nicotinamide adenine dinucleotide (NAD<sup>+</sup>), resazurin, phenylmethanesulfonyl fluoride (PMSF), dithiothreitol (DTT), Triton X-100, streptomycin sulfate, imidazole, pyrophosphatase (PPase, organism/activity?), glycerol, and Amicon® centrifugal filters were purchased from Sigma Aldrich. Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), sodium phosphite dibasic pentahydrate (Phi), D-(+)-glucose, LB medium (Miller, powder), malt extract, and yeast extract were purchased from Thermo Fisher Scientific. Formaldehyde (37% solution) and hydrochloric acid (36.5-38.0% solution) were purchased from VWR. Kanamycin was purchased from Genesee Scientific. Ni-NTA resin was purchased from Qiagen. Phosphite dehydrogenase (PtxD) was available from a previous study.<sup>17</sup>

*Streptomyces viridochromogenes* DSM 40736 was obtained from the Leibniz Institute DSMZ (Braunschweig, Germany). Mutant strains WM6547, WM6601, WM6602, WM6548, WM6546, and WM6604 ( $\Delta$ phpC,  $\Delta$ phpD,  $\Delta$ phpE,  $\Delta$ phpJ,  $\Delta$ phpF, and  $\Delta$ phpH, respectively) were a gracious gift from William W. Metcalf.<sup>14</sup>

## **Growth and Sampling of *Streptomyces viridochromogenes* DSM 40736**

*S. viridochromogenes* colonies were transferred to 15 mL of maltose-yeast-glucose (MYG) medium (pH 6.5) and shaken at 30 °C for 2 days. These cultures were then transferred to 100 mL of MYG (with 0.1 mM or 0.5 mM PF, where indicated) with 5 mm glass beads (Sigma Aldrich) and shaken at 30 °C for four days. Culture supernatant was collected by removing 50 mL of culture and centrifuging at  $10,000 \times g$  for 10 min. As a negative control, 100 mL of 0.1 mM PF in sterile MYG was incubated similarly and sampled in parallel to the live cultures. The supernatant was decanted into fresh 50 mL centrifuge tubes, and the pellet and supernatant were frozen in liquid nitrogen. The supernatant was lyophilized to a powder, then resuspended in 1 mL Milli-Q H<sub>2</sub>O. Prior to NMR analysis, 1 mM TSP and 10% D<sub>2</sub>O were added for internal reference.

## **NMR Analysis**

NMR analysis was done at 25 °C on a Bruker Avance NEO 500 MHz spectrometer fitted with a 5 mm CryoProbe Prodigy BBO probe and acquired using TopSpin 4.1.4 software. After sample introduction, <sup>1</sup>H-NMR, proton-decoupled <sup>31</sup>P-NMR, and proton-coupled <sup>31</sup>P-NMR spectra were collected. <sup>1</sup>H-NMR spectra were collected using ‘zgesgp’, which features water suppression using excitation sculpting with gradients.<sup>18</sup> Proton-decoupled <sup>31</sup>P-NMR spectra were collected with ‘P31CPD’ featuring a 2 s recycle delay, averaging 64 scans unless otherwise specified. Proton-coupled <sup>31</sup>P-NMR spectra were collected with ‘P31’ using a 2 s recycle delay, averaging 100 scans unless otherwise specified. The <sup>1</sup>H peak of TSP was used as the 0.0 ppm reference. The decoupled or coupled <sup>31</sup>P-NMR spectra were indirectly referenced using the TSP signal relative to 85% H<sub>3</sub>PO<sub>4</sub>.<sup>19</sup> Spectral data were processed with MestReNova software.

### **Quantification of Phosphite**

Phosphite concentrations were determined using a fluorometric assay coupled to the activity of PtxD, as described previously.<sup>17</sup> *S. viridochromogenes* spent supernatant was supplied directly to the assay without modification. Enzymatic reactions were passed through a 3 kDa Amicon® Ultra-0.5 or 3 kDa Amicon® Ultra-15 centrifugal filter unit (EMD Millipore) at  $14,000 \times g$  or  $5,000 \times g$ , respectively, for 30 min.

### ***Streptomyces viridochromogenes* $\Delta$ phpH Lysate Preparation**

*S. viridochromogenes*  $\Delta$ phpH deletion mutant was grown in MYG liquid medium as described above. After 4 days of incubation the cells were collected and frozen in liquid nitrogen. The cells were later thawed and resuspended in lysis buffer (20 mM MES, 100 mM NaCl, 10 mM PF, 1% Triton X-100, 1 mM PMSF, and 2 mM DTT at pH 6.5) at a ratio of 4 mL buffer per g cell pellet. Cells were lysed via ultrasonication (12 min processing time with a 1/4" probe, 10 s on-10 s off, at 60% amplitude) and insoluble cellular material was removed by centrifuging at  $14,000 \times g$  for 10 min. The lysate was split into 3 x 12.5 mL fractions, to which 5 mM ATP (from a 100 mM stock, pH 6.5) and 10 U PPase, 5 mM CTP (from a 100 mM stock, pH 6.5) and 10 U PPase, or 10 U PPase were added. The lysates were shaken at 200 rpm at 37 °C for 24 h. Afterwards, the lysates were passed through a 3 kDa centrifugal filter at  $5,000 \times g$  and the flow-through was collected. Of the 12 mL collected, 2 mL were reserved for Phi quantification and the remaining 10 mL were frozen in liquid nitrogen and lyophilized to a powder. For <sup>31</sup>P-NMR analysis, the powder was resuspended in 0.5 mL MilliQ H<sub>2</sub>O and supplied with 1 mM TSP and 10% D<sub>2</sub>O. Prior to use in the phosphite assay, cell lysates were diluted 1.5-fold to minimize medium effects.

### **Over-expression and Purification of His-SUMO-PhpF**

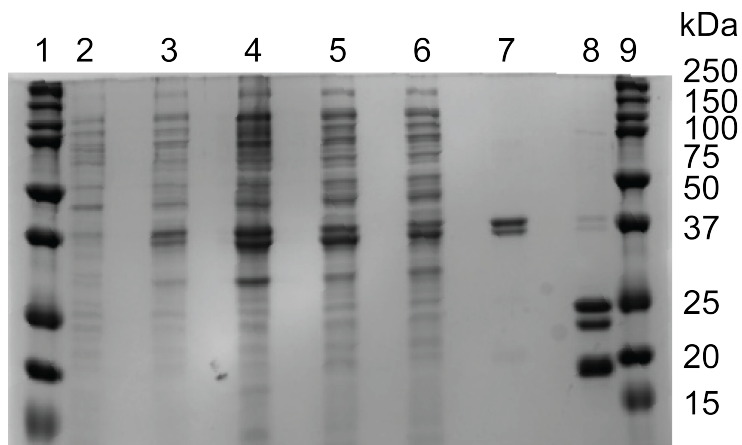


The pET-His-SUMO-PhpF plasmid was constructed using HiFi Assembly. PCR with Phusion High-Fidelity DNA Polymerase (New England Biolabs, USA) was used to generate overlapping ends for His-Smt3-NpuDnaEN102-CBD<sup>20</sup> backbone and the *phpF* gene fragment. PCR products were digested with DpnI (New England Biolabs, USA) overnight at 37°C. Fragments and backbones with overlapping ends were ligated using HiFi DNA Assembly Master Mix (New England Biolabs). The resulting DNA product was transformed into *E. coli* DH5 $\alpha$  cells and the assembly of the pET-His-SUMO-PhpF plasmid was verified by Sanger DNA sequencing.

*E. coli* BL21(DE3) cells were transformed with the pET-His-SUMO-PhpF plasmid, and were cultured overnight in 10 mL LB media with 25  $\mu$ g/mL kanamycin. Saturated cultures were diluted at a 1:100 ratio in fresh LB with 25  $\mu$ g/mL kanamycin, grown to an OD<sub>600</sub> of 0.6-0.9, induced with 0.1 mM IPTG, and incubated at 23°C for 20 h. The resulting cell pellet was resuspended in Buffer A (20 mM Tris buffer, 500 mM NaCl, and 10% v/v glycerol at pH 7.6) with 4 mL buffer per g cell pellet. The cell pellet was homogenized and lysed using a pre-cooled Avestin C3 French Press at 14,000 psi in one pass. The resulting lysate was centrifuged at  $14,636 \times g$  for 10 min. DNA was precipitated from the supernatant by the addition of 0.2 volume equivalents of 6% w/v streptomycin sulfate, followed by centrifugation at  $14,636 \times g$  for 30 min. The supernatant was then loaded on a Ni-NTA column pre-equilibrated with Buffer A. The column was washed with 30 CV of Buffer B (Buffer A supplemented with 50 mM imidazole), then the desired protein was eluted with 3 CV of Buffer C (Buffer A supplemented with 500 mM imidazole). Protein-containing fractions were combined and concentrated in a 10 kDa Amicon® Ultra-15 centrifugal filter unit at  $5,000 \times g$  for 10 min. The protein concentrate was buffer exchanged with Buffer D (20 mM MOPS, 100 mM KCl, and 10% v/v

glycerol at pH 7.6) using a HiTrap Desalting 5 mL column containing Sephadex G-25 resin. The resulting sample was assessed for purity using SDS-PAGE (**Figure 13**).

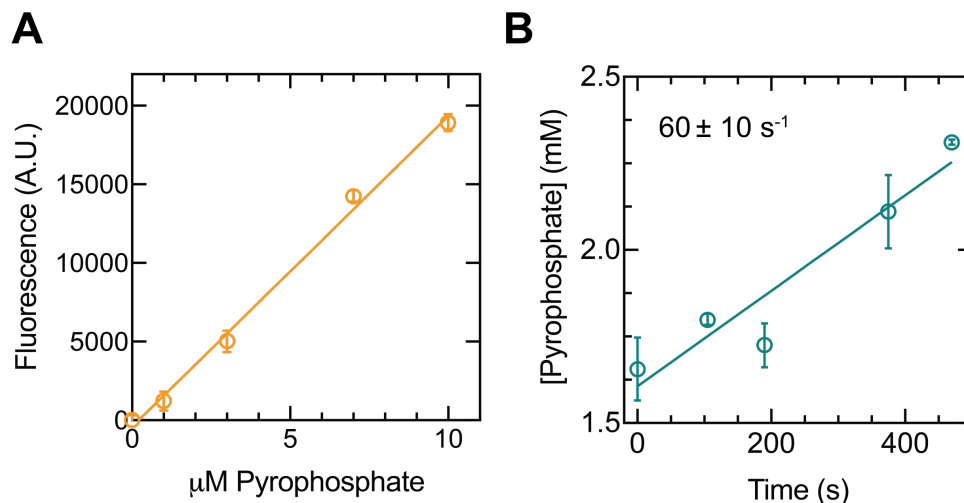
Enzyme activity was determined using a pyrophosphate assay kit (Sigma Aldrich). Enzymatic reactions were prepared in 1.2 mL reactions with 50 mM MES (pH 6), 10 mM MgCl<sub>2</sub>, 5 mM PF, 15 mM CTP, and 1 µg His-SUMO-phpF. Aliquots were sampled throughout incubation at 37°C by passing 200 µL of the reaction mix through a 10 kDa Amicon® Ultra-15 centrifugal filter unit at 14,000 × g for 20 s. The filtrate was diluted 1:100 before use in the pyrophosphate assay (**Figure 14**).



**Figure 13.** SDS-PAGE gel describing the purification of His-SUMO-phpF. Lanes contain the *E. coli* BL21(DE3) pellet with prior to induction (2), the *E. coli* BL21(DE3) pellet with overexpressed His-SUMO-phpF (3), the cell pellet after lysis (4), the cell lysate (5), Ni-NTA column flow-through (6), eluted final protein (7), and ULP1-digested protein product (8) and a molecular weight ladders (1,9). The His-SUMO-phpF theoretical mass is 35444 Da.

### His-SUMO-phpF Enzymatic Assays

Native activity of phpF was verified in 2 mL assay reactions containing 50 mM HEPES (pH 7.25), 10 mM MgCl<sub>2</sub>, 1 mM CTP, and 1 mM PF as reported previously.<sup>14</sup> To initiate the reaction, 5 µg of His-SUMO-phpF and 5 U PPase were added. The reaction was incubated at 37°C for one hour before analysis by <sup>31</sup>P-NMR. To observe Phi-producing reactivity, 4 mL



**Figure 14.** Quantifying His-SUMO-phpF activity by assaying for pyrophosphate. **A** Standard curve showing fluorescence response in the linear range of the pyrophosphate assay. **B** Example kinetics experiment for the production of pyrophosphate during the formation of phosphonoformyl-CMP from phosphonoformate and CTP.

reaction mixes were prepared with 50 mM MES (pH 6 or 6.5), 10 mM MgCl<sub>2</sub>, 5 mM PF, 15 mM CTP, 20 μg His-SUMO-phpF, and 10 U PPase, and were incubated at 37°C. Aliquots were removed throughout the reaction for analysis with NMR and/or the fluorescence-based Phi assay. To determine if conversion to Phi was dependent on the presence of active enzyme, a 1 mL reaction mix was prepared with 50 mM MES (pH 6), 10 mM MgCl<sub>2</sub>, 5 mM PF, 15 mM CTP, 5 μg His-SUMO-phpF, and 2.5 U PPase. The reaction was allowed to proceed for 2 h, after which 1 μL of trypsin (0.5 mg/mL, Promega) was added and 0.5 mL of the reaction mix was removed and immediately stored at -20°C. The remaining 0.5 mL of the reaction was incubated at 37°C for 24 h. Both trypsin-containing samples were then analyzed with NMR.

### ATMP Synthesis

The synthesis of ATMP was adapted from reported methods.<sup>21</sup> *S. viridochromogenes* cultures were grown as previously described, but with the addition of 5 mM PF to culture medium and spent media collection after just 2 days of growth. Spent medium (400 mL) was

collected in 50 mL Falcon tubes, lyophilized to a powder, resuspended in 4 mL MilliQ H<sub>2</sub>O and spun down at  $8,000 \times g$  to remove all insoluble material. The resulting 3.5 mL was placed in a three-necked round bottom flask with 3.5 mL concentrated (37%) HCl and 0.63 g NH<sub>4</sub>Cl. The reaction mixture was brought to reflux temperature, then 5.6 mL of formaldehyde was added dropwise over the course of 1 h. The reaction was refluxed for an additional 1 h, then was left at room temperature overnight. The products were precipitated by adding acetone to a 5:1 acetone-water ratio. The precipitate was vacuum filtered and products were verified with <sup>31</sup>P-NMR. Due to low signal intensity, phase correction was adjusted manually to ensure all absorption was positive. To increase the signal-to-noise ratio, the apodization exponential function was adjusted from 1 Hz to 3 Hz.

Enzyme activity was determined using a pyrophosphate assay kit (Sigma Aldrich). Enzymatic reactions were prepared in 1.2 mL reactions with 50 mM MES (pH 6), 10 mM MgCl<sub>2</sub>, 5 mM PF, 15 mM CTP, and 1 µg His-SUMO-phpF. Aliquots were sampled throughout incubation at 37°C by passing 200 µL of the reaction mix through a 10 kDa Amicon® Ultra-15 centrifugal filter unit at  $14,000 \times g$  for 20 s. The filtrate was diluted 1:100 before use in the pyrophosphate assay (**Figure 14**).

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mM CTP, 20  $\mu$ g His-SUMO-phpF, and 10 U PPase, and were incubated at 37°C. Aliquots were removed throughout the reaction for analysis with NMR and/or the fluorescence-based Phi assay. To determine if conversion to Phi was dependent on the presence of active enzyme, a 1 mL reaction mix was prepared with 50 mM MES (pH 6), 10 mM MgCl<sub>2</sub>, 5 mM PF, 15 mM CTP, 5  $\mu$ g His-SUMO-phpF, and 2.5 U PPase. The reaction was allowed to proceed for 2 h, after which 1  $\mu$ L of trypsin (0.5 mg/mL, Promega) was added and 0.5 mL of the reaction mix was removed and immediately stored at -20°C. The remaining 0.5 mL of the reaction was incubated at 37°C for 24 h. Both trypsin-containing samples were then analyzed with NMR.

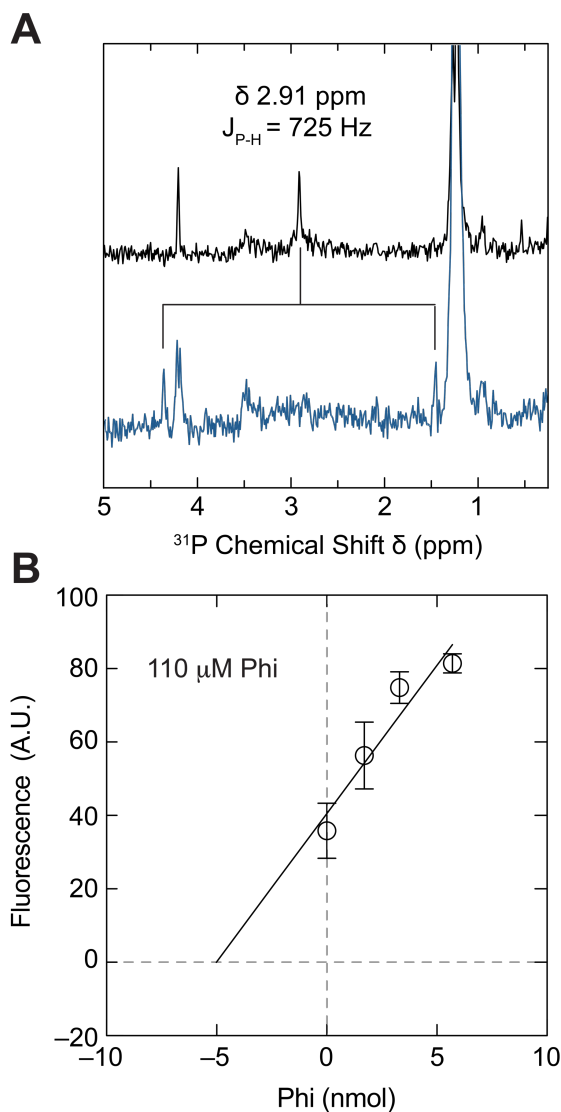
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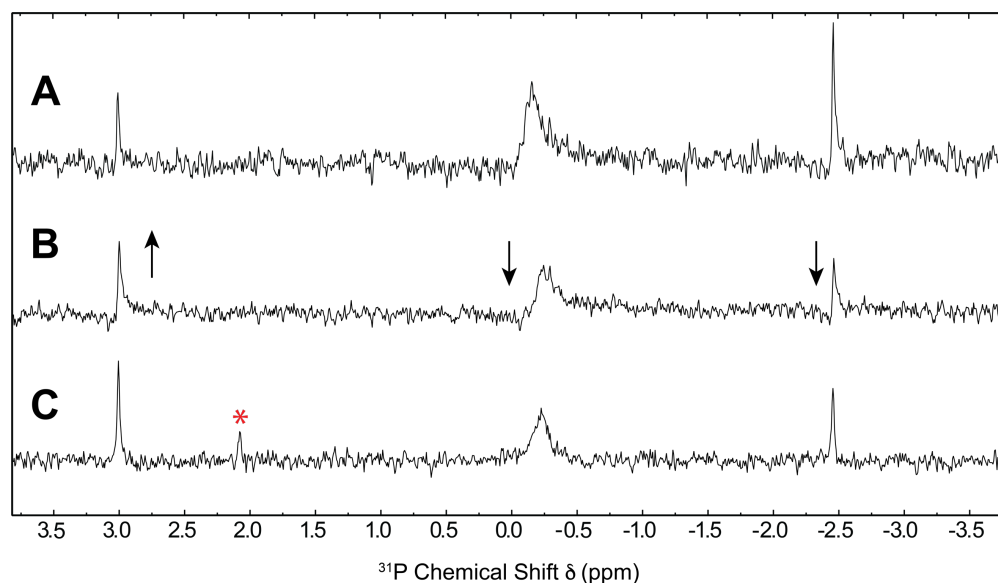
## Results and Discussion

### Phi Production by *S. viridochromogenes*

Previous studies on the  $\Delta phpF$  strain of *S. viridochromogenes*, grown on MYG media, suggested that Phi was produced via the accumulation of the PhpF substrate, PF, and its



**Figure 15.** Phi signal in *S. viridochromogenes* spent medium. **A** Proton-decoupled (black) and proton-coupled (blue)  $^{31}\text{P}$ -NMR spectra of *S. viridochromogenes* spent medium after 4 days of incubation at 30°C in MYG with 500  $\mu\text{M}$  PF, concentrated 100-fold. Splitting of the Phi peak at 2.91 ppm is shown along with the coupling constant,  $J_{P-H}$ . **B** Quantification of Phi in *S. viridochromogenes* unconcentrated spent medium using the Phi assay. The standard addition curve reading was  $110 \pm 40$   $\mu\text{M}$  Phi.

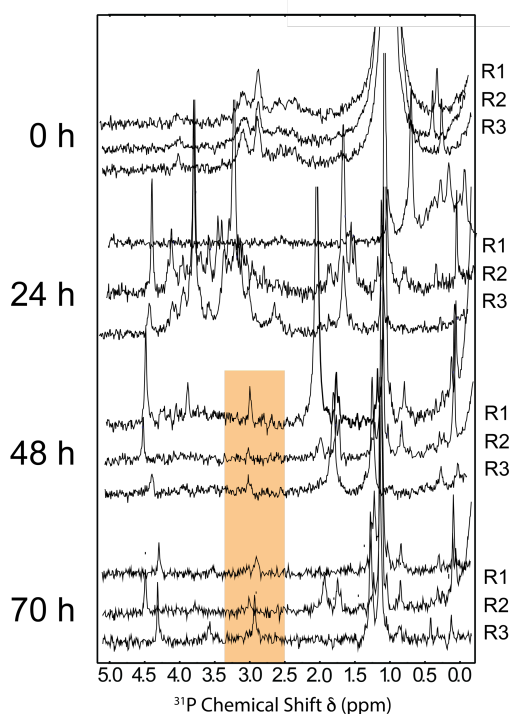


**Figure 16.** Phi signal confirmation with Phi- and Pi-spiking. **A** Proton-decoupled  $^{31}\text{P}$ -NMR spectra of *S. viridochromogenes* spent medium after 4 days of growth in MYG with 100  $\mu\text{M}$  PF, concentrated 100-fold. Spectrum was acquired with 48 scans. **B** Sample from A spiked with 200  $\mu\text{M}$  Phi. Spectrum was acquired with 24 scans. Signals indicated with a downward-facing arrow decreased in peak area by 53-54%. Putative Phi signal indicated with an upward-facing arrow increased in peak area by 49%. **C** Sample from B spiked with 200  $\mu\text{M}$  Pi. The appearance of a new peak at 2.08 ppm is indicated with an asterisk.

spontaneous decarboxylation to Phi.<sup>14</sup> To replicate and potentially enhance Phi production, we cultivated *S. viridochromogenes* in MYG medium supplemented with 500  $\mu\text{M}$  PF.<sup>14</sup> After 4 days of growth we observed a putative Phi peak at 2.91 ppm by  $^{31}\text{P}$ -NMR (**Figure 15A**). The identity of the Phi peak was supported by  $^1\text{H}$ -coupled  $^{31}\text{P}$ -NMR, with a characteristically large  $^1\text{H}$  coupling constants (725 Hz), indicative of a direct P-H bond, and by comparison with an authentic Phi standard in aqueous solution (**Supplemental Information Figure 2.1**).<sup>22</sup> Minor differences in chemical shift and  $^1\text{H}$ - $^{31}\text{P}$  coupling strength between the Phi signal from *S. viridochromogenes* media and the authentic standard are expected and reasonable based on the medium-dependence of the ionizable Phi.<sup>23–25</sup> We further confirmed the identity of the  $^{31}\text{P}$ -NMR signal by spiking Phi and  $\text{P}_i$  into the spent media sample, demonstrating that the peak was indeed Phi and not attributable to inorganic  $\text{P}_i$  (**Figure 16**). Using our previously

developed enzyme-coupled fluorometric assay, we quantified the Phi product.<sup>17</sup> The cell culture medium, following growth, contained  $110 \mu\text{M} \pm 40 \mu\text{M}$  Phi (**Figure 15B**), indicating a near quantitative conversion of PF to Phi in culture. When PF was incubated in sterile MYG medium, no Phi was observed, either by  $^{31}\text{P}$ -NMR or the enzymatic Phi assay (**Supplemental Information Figure 2.2**). These results confirm that PF does not spontaneously decompose to Phi over the time scale, temperature, and pH of the *S. viridochromogenes* cultures.<sup>16</sup>

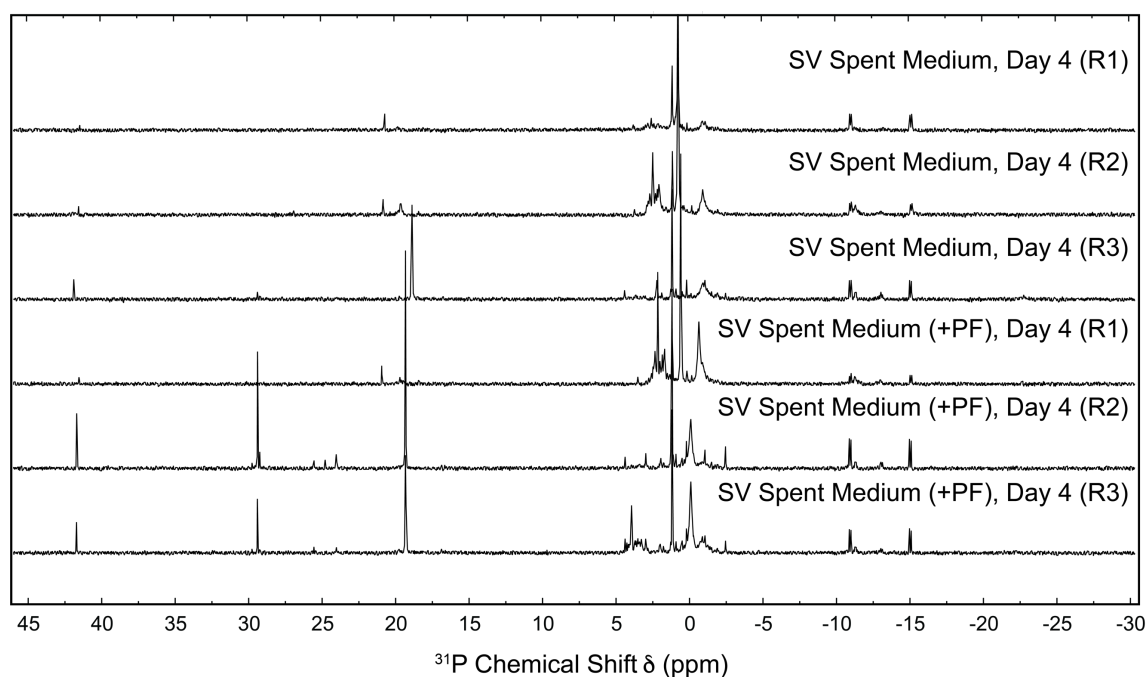
We observe a Phi signal in the culture supernatant as early as 48 h after inoculation, which persisted through the remaining 2 days of culturing (**Figure 17**). At the same 48 h time point, we also detected other phosphonate intermediates of the PTT pathway in the spent medium supernatant. To determine the dependence of the Phi signal on supplemented PF, *S.*



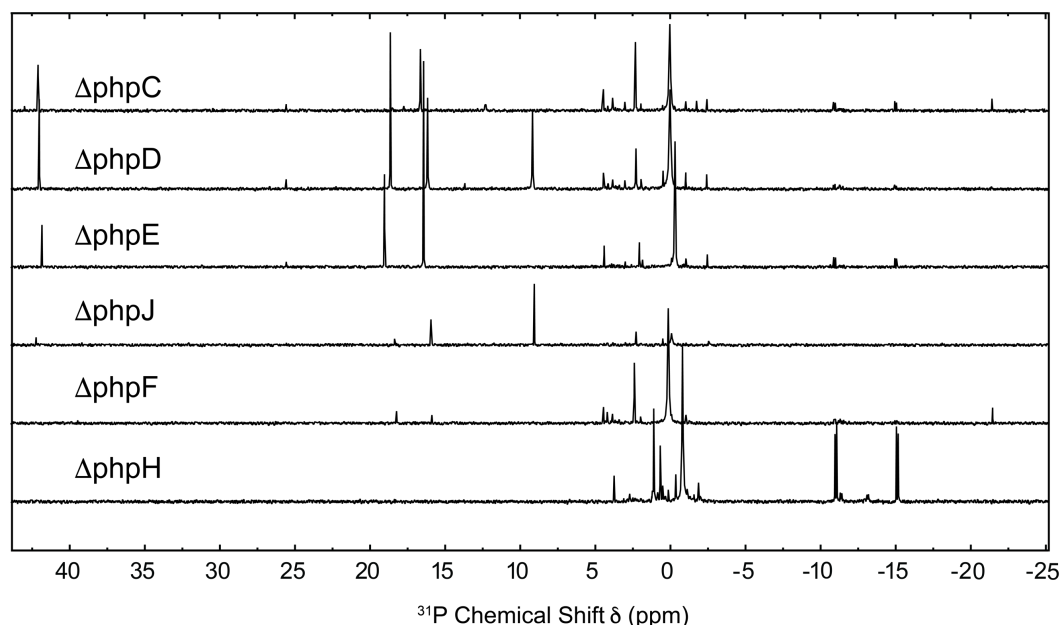
**Figure 17.** Proton-decoupled  $^{31}\text{P}$ -NMR spectrum of *S. viridochromogenes* spent media sampled throughout incubation. Cultures were grown at  $30^\circ\text{C}$  in MYG with  $500 \mu\text{M}$  PF, and the spent medium was concentrated 100-fold. Three biological replicates are shown for each timepoint. Putative Phi peaks are highlighted.



*viridochromogenes* cultures were grown with and without 500  $\mu$ M PF in MYG medium. In all biological replicates, we detected Phi only in cultures supplied with PF (**Figure 18**). The supplementation of PF also increased the quantity of several phosphonate intermediates in the PTT pathway ( $\delta$  15-45 ppm), suggesting that the PTT biosynthetic genes are expressed and that PTT production is regulated via the production of a PF precursor.<sup>26</sup> This correlation between Phi and phosphonate production implicates the PTT pathway in Phi biosynthesis but does not establish molecular specificity.



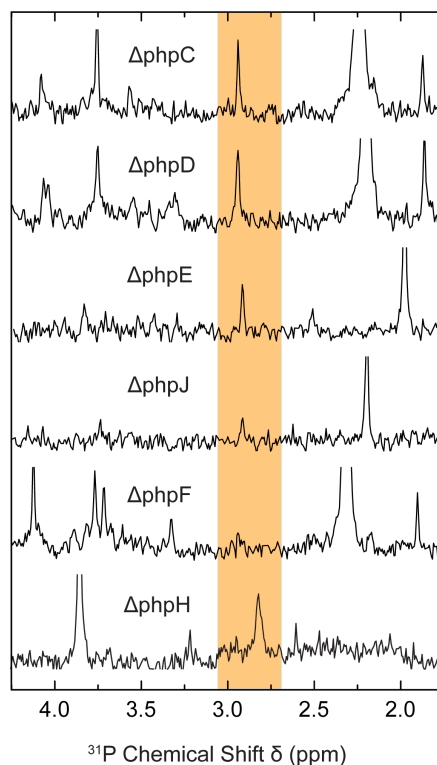
**Figure 18.** Phi signal dependence on PF in *S. viridochromogenes* spent medium. Proton-decoupled  $^{31}\text{P}$ -NMR spectrum of *S. viridochromogenes* spent medium after 4 days of incubation at 30°C in MYG with (bottom) or without (top) 500  $\mu$ M PF, concentrated 100-fold. Three biological replicates are shown (R1-R3).



**Figure 19.** Proton-decoupled  $^{31}\text{P}$ -NMR spectra of *S. viridochromogenes* deletion mutants. Cultures were grown in MYG with 500  $\mu\text{M}$  PF for 4 days and the spent media was concentrated 100-fold prior to analysis. The  $\Delta\text{phpC}$  mutant shows the expected accumulation of 2-aminomethylphosphonate ( $\delta$  16.7), the  $\Delta\text{phpE}$  and  $\Delta\text{phpD}$  mutants similarly show expected intermediate accumulation (hydroxymethyl phosphonate,  $\delta$  16.7, and hydroxyethyl phosphonate,  $\delta$  18.7, respectively) and Pt production. The  $\Delta\text{phpH}$  mutant shows no accumulation of any intermediate in the PTT biosynthesis pathway, in accordance with the results of Blodgett *et al.*

### *S. viridochromogenes* Deletion Mutants

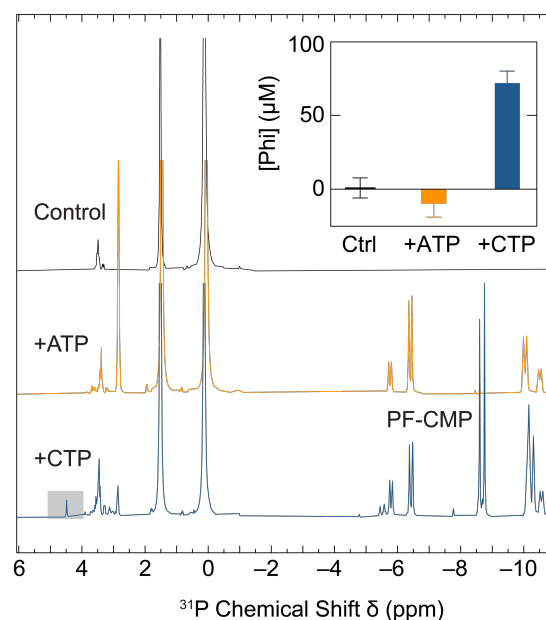
We analyzed the spent media of *S. viridochromogenes* mutants with genetic deletions in the PTT biosynthetic pathway ( $\Delta\text{phpC}$ ,  $\Delta\text{phpD}$ ,  $\Delta\text{phpE}$ ,  $\Delta\text{phpJ}$ ,  $\Delta\text{phpF}$ , and  $\Delta\text{phpH}$ ), supplemented with 500  $\mu\text{M}$  PF, to determine if any of these genes contributed to Phi production. As expected, deletions in the *phn* operon of genes involved in the biosynthesis of the PF intermediate ( $\Delta\text{phpC}$ ,  $\Delta\text{phpD}$ ,  $\Delta\text{phpE}$ , and  $\Delta\text{phpJ}$ ) did not prevent the production of PTT ( $\delta$  42 ppm) from exogenously supplied PF. Conversely, deletions of genes downstream from PF synthesis ( $\Delta\text{phpF}$  and  $\Delta\text{phpH}$ ) showed no PTT signal (**Figure 19**). Unexpectedly, all deletion mutants except for  $\Delta\text{phpF}$  show Phi signal (**Figure 20**). Previous studies on the



**Figure 20.** Proton-decoupled  $^{31}\text{P}$ -NMR spectrum of *S. viridochromogenes* deletion mutant spent media after 4 days of incubation at 30°C in MYG with 100  $\mu\text{M}$  PF, concentrated 100-fold. Putative Phi peaks are highlighted.

*ΔphpF S. viridochromogenes* mutant identified a peak by  $^{31}\text{P}$ -NMR that was attributed to Phi. We also observed a  $^{31}\text{P}$ -NMR peak in *ΔphpF* growth medium supernatant in the vicinity of Phi signals ( $\delta$  4.45 ppm); however, this species did not exhibit the characteristically large  $^1\text{H}$ - $^{31}\text{P}$  coupling of Phi in the  $^1\text{H}$ -coupled  $^{31}\text{P}$ -NMR ( $J_{\text{H-P}} = \sim 10$  Hz), suggesting it is a phosphonate, but not Phi. Based on these genetic knockout studies, the molecular origins of Phi appear to be a result of PhpF activity in *S. viridochromogenes* (**Scheme 4**).

To separate Phi production from cellular growth and confirm the Phi signal's dependence on enzymatic activity, we assayed the soluble lysate of *S. viridochromogenes*  $\Delta phpH$  for Phi production. When supplemented with 10 mM PF, the lysate produced Phi, but only in the presence of PPase and CTP (**Figure 21**). Although spectral congestion prevented the identification of Phi by  $^1\text{H}$ -decoupled  $^{31}\text{P}$ -NMR,  $^1\text{H}$ -coupled  $^{31}\text{P}$ -NMR revealed an emergent peak corresponding to the Phi down-field peak of the doublet at 4.48 ppm, as well as signals consistent with phosphonoformyl-CMP (PF-CMP, **Figure 21**).<sup>14</sup> To confirm the Phi signal, we quantified the Phi concentration using the enzymatic Phi assay, which showed  $73 \pm 7 \mu\text{M}$  Phi in the CTP-supplemented lysate and no Phi production in the absence of PPase or CTP (**Figure 21, inset**). The proposed function of PhpF is that of a CTP-phosphonoformate

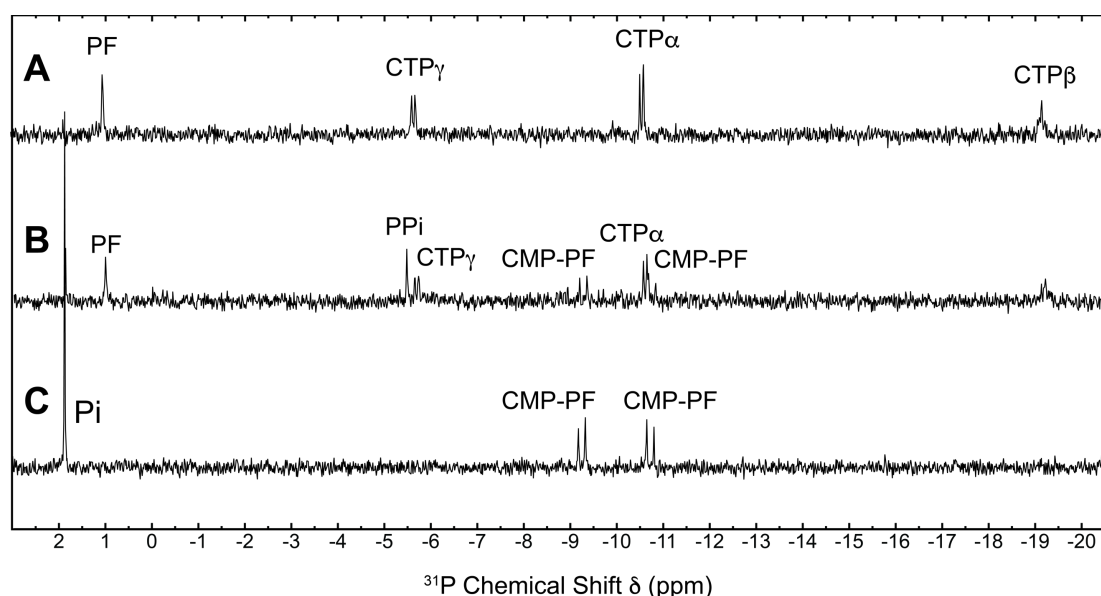


**Figure 21.** Phi production in *S. viridochromogenes*  $\Delta phpH$  lysate. Proton-coupled  $^{31}\text{P}$ -NMR spectra of lysate incubated with 10 U PPase (control), 10 mM ATP and 10 U PPase, or 10 mM CTP and 10 U PPase. A putative Phi doublet peak at 4.48 ppm is highlighted. Inset: Quantification of Phi in cell lysates using the Phi assay.

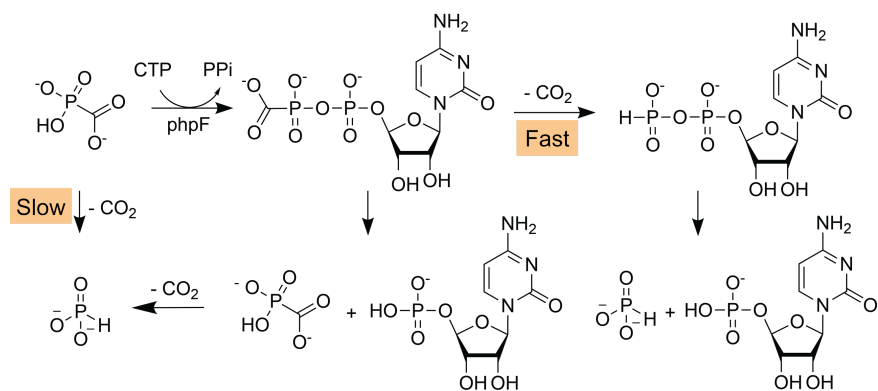
nucleotidyltransferase. The concurrent production of PF-CMP and Phi suggested that PhpF is responsible for the formation of Phi via a PF-CMP intermediate.<sup>27</sup>

### Enzymatic Assays with Recombinant His-SUMO-phpF

We hypothesized that activation of PF by PhpF promotes the decarboxylation of PF-CMP, yielding a phosphinated intermediate (Phi-CMP) that could decompose to Phi and CMP upon the hydrolysis of the phosphoanhydride bond (**Scheme 5**). To test this hypothesis, we recombinantly expressed and purified PhpF from *E. coli* for analysis. The enzyme exhibited the expected reactivity in the presence of PF, CTP, MgCl<sub>2</sub>, and PPase, fully converting PF to PF-CMP over 1 h at 37°C (**Figure 22**). To counteract PF-CMP hydrolysis to CMP and PF, we added excess CTP to the reaction mixture, and carried out the reaction at pH 6 to favor

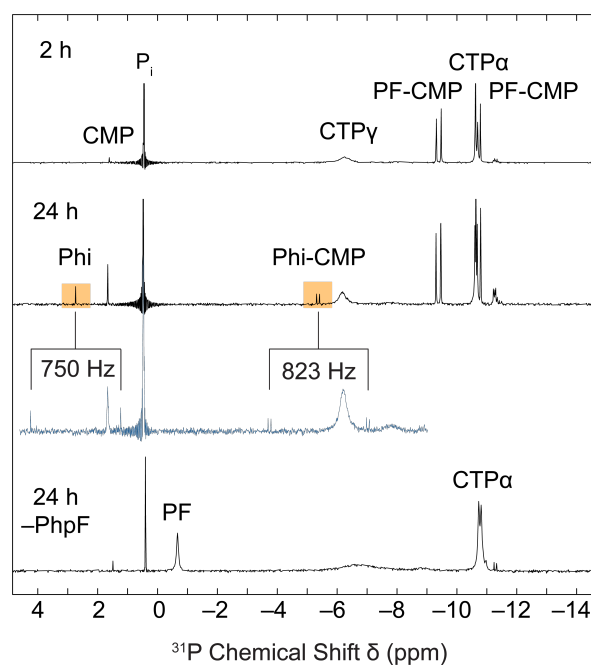


**Figure 22.** Proton-decoupled <sup>31</sup>P-NMR spectra confirming CTP-phosphonoformate nucleotidyltransferase activity of His-SUMO-phpF. **A** Assay reaction mixture containing 50 mM HEPES (pH 7.25), 10 mM MgCl<sub>2</sub>, 1 mM CTP, and 1 mM PF. **B** Assay reaction mixture after addition of 5 μg His-SUMO-phpF and incubation at 37°C for 1 h. **C** Assay reaction mixture after addition of 5 μg His-SUMO-phpF and 5 U PPase and incubation at 37°C for 1 h.

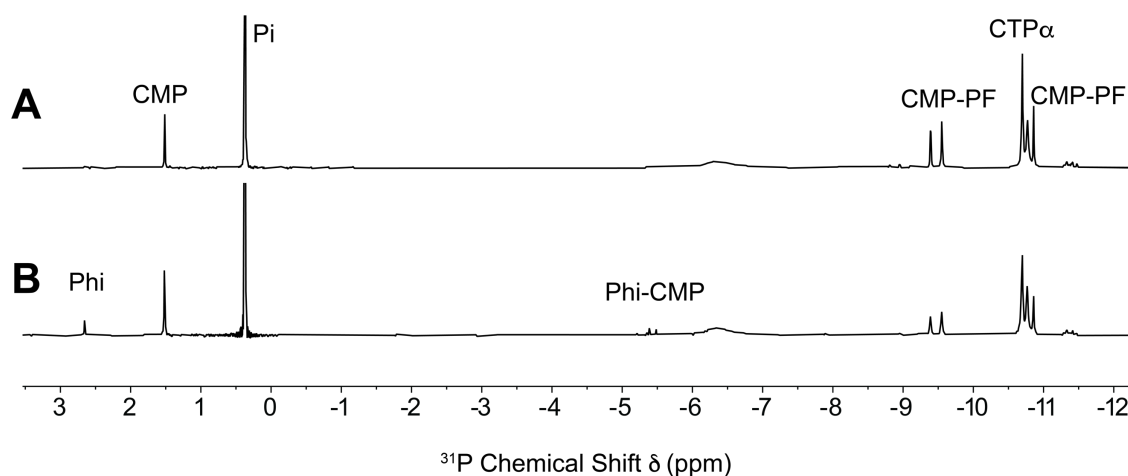


**Scheme 5.** Proposed mechanism for the formation of Phi by *S. viridochromogenes*.

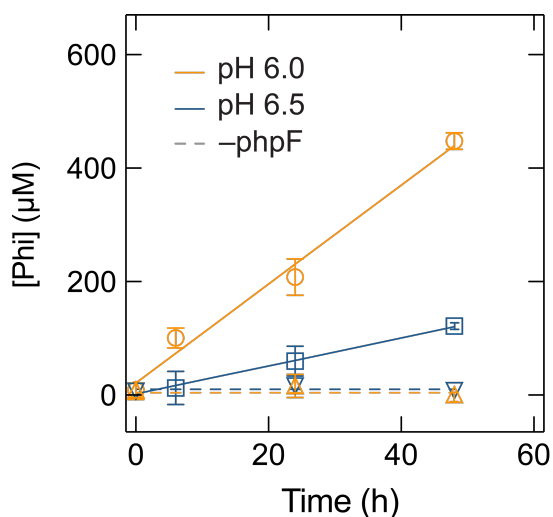
decarboxylation by CMP-PF. After 2 h of incubation at 37°C, we observed PF-CMP, CTP, and P<sub>i</sub> (**Figure 23**, top). After 24 h of incubation, we observed two new peaks, a singlet at 2.74 ppm and a doublet at 5.36 ppm with a splitting of 750 Hz and 823 Hz, respectively (**Figure 23**, middle) that were not present in the PhpF-free control after 24 h (**Figure 23**, bottom). Based on the chemical shift and J<sub>P-H</sub>, we assign the 2.74 ppm peak to Phi, and the -5.36 ppm doublet to Phi-CMP. To determine if the conversion of PF-CMP to Phi-CMP was enzymatic or spontaneous, we treated the reaction with trypsin after 2 h of incubation to digest PhpF. The digested sample still produced Phi from PF-CMP over 24 h (**Figure 24**). We again quantified the Phi production rate using the enzymatic Phi assay (**Figure 25**). At pH 6, Phi was produced at a rate of 8.7 μM Phi/h. Given the relatively high activity of PhpF for its native reaction ( $k_{\text{cat}}$  of  $60 \pm 10 \text{ s}^{-1}$ ) and the observation of Phi production from trypsin-digested enzyme assays (**Figure 14**), Phi production appears to proceed nonenzymatically from the decomposition of Phi-CMP. Given the prevalence of cytidyltransferase activation in phosphonate biosynthesis,<sup>28</sup> these reactive intermediates may play a larger role in metabolite leakage in the environment.



**Figure 23.** Phi production in phpF enzymatic assays. Proton-decoupled  $^{31}\text{P}$ -NMR spectra of a phpF assay reaction (50 mM MES, pH 6, 10 mM  $\text{MgCl}_2$ , 5 mM PF, 15 mM CTP, 5  $\mu\text{g/mL}$  His-SUMO-phpF, and 5 U/mL PPase) after 2 h and 24 h (black spectra, top) incubation at  $37^\circ\text{C}$ . The proton-coupled  $^{31}\text{P}$ -NMR spectrum of the reaction after 24 h is shown in blue. A proton-decoupled  $^{31}\text{P}$ -NMR spectrum of a control reaction without His-SUMO-phpF after 24 h incubation is shown (bottom).



**Figure 24.** Proton-decoupled  $^{31}\text{P}$ -NMR spectra demonstrating Phi production in trypsin-digested His-SUMO-phpF reaction mixtures. **A** Reaction mixture containing 50 mM MES (pH 6), 10 mM  $\text{MgCl}_2$ , 5 mM PF, 15 mM CTP, 5  $\mu\text{g}$  His-SUMO-phpF, and 2.5 U PPase incubated for 2 h before addition of 1  $\mu\text{L}$  trypsin. **B** Trypsin-digested sample after 24 h incubation at  $37^\circ\text{C}$ .

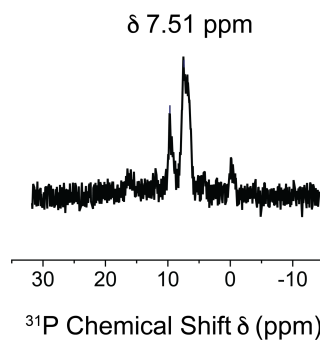


**Figure 25.** Conversion of PF to Phi by His-SUMO-phpF activity. The Phi yield with time of the phpF assay reaction is shown at pH 6 (orange) and pH 6.5 (blue). Reactions where His-SUMO-phpF was inactivated by heat before addition to the reaction mixture are shown with triangles and dashed lines.

## ATMP Synthesis

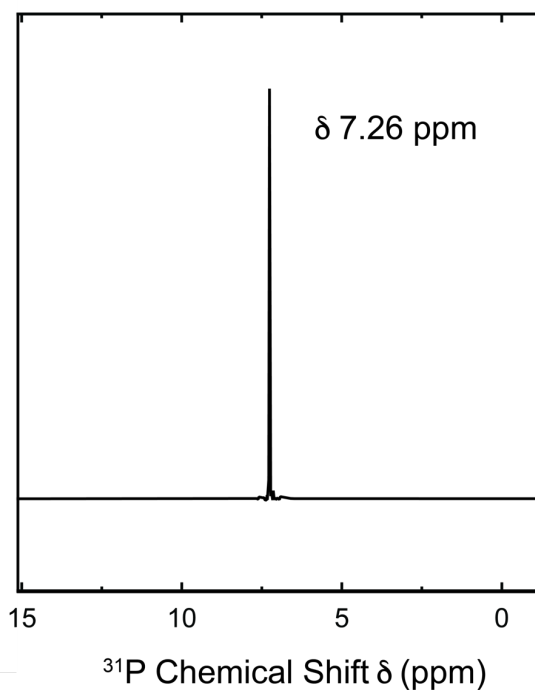
ATMP is a metal chelator with a wide range of industrial applications including the prevention of scaling, corrosion, and use in detergents.<sup>29–31</sup> ATMP can be produced through a facile one-pot Mannich-type reaction involving phosphorous acid, formaldehydes, and ammonia.<sup>21</sup> To demonstrate the utility of *S. viridochromogenes* in P activation, we used Phi-rich spent media as a feedstock for a hybrid biological-abiological synthesis of ATMP. To optimize phosphonate synthesis, and consequently Phi production, by *S. viridochromogenes*, we grew cultures with an order of magnitude greater PF concentration in the medium (5 mM). The resulting concentrated spent media was able to provide phosphorous acid in the ATMP synthesis reaction, resulting in a <sup>31</sup>P-NMR peak at 7.51 ppm (**Figure 26**). This is in good agreement with literature values of 7.8 ppm<sup>32</sup> and our own analysis of an ATMP standard (7.26 ppm, **Figure 27**). While the overall yield was modest from 400 mL of culture (<5 mg), this proof-of-concept synthesis demonstrates that *S. viridochromogenes* cultures can serve as a





**Figure 27.** Preparation of ATMP from *S. viridochromogenes* spent medium. ATMP synthesis crude product was resuspended in 10%  $\text{D}_2\text{O}$  and analyzed with proton-decoupled  $^{31}\text{P}$ -NMR. The major product and putative ATMP peak is labeled.

feedstock for the synthesis of industrially relevant reduced phosphorus products. Furthermore, optimization of the biosynthetic pathway to both PF and PF-CMP from  $\text{P}_i$  and integration into



**Figure 26.** Proton-decoupled  $^{31}\text{P}$ -NMR spectra of aminotris(methylenephosphonic acid) (ATMP) standard.

a more atom and energy-efficient host system is expected to drastically improve biotechnological access to diverse phosphochemicals via microbially produced Phi.

## Conclusion

Microbial Phi production presents a promising alternative to energy-intensive legacy methods. Here we show Phi production in *S. viridochromogenes* as a byproduct of the PTT biosynthesis pathway through the activity of the PF-activating enzyme PhpF. In addition, we show that *S. viridochromogenes* culture can serve as a feedstock for the industrially relevant chemical synthesis of ATMP. The characterization of a Phi-producing pathway in microbial metabolism opens the potential for discovery of other microbial means of Phi generation, perhaps similarly related to phosphonate metabolism. Further work in microbial Phi production will have significant implications for sustainable reduced P reduction as well as the global biogeochemical P cycle.

## Acknowledgements

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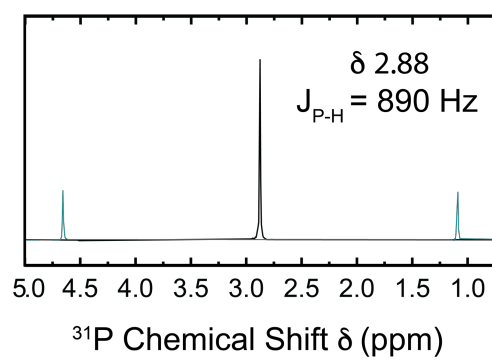
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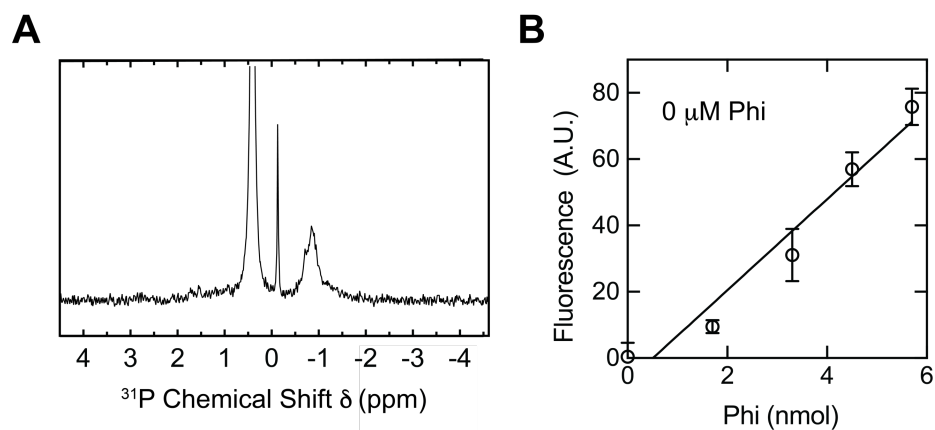
## Supplemental Information

### LIST OF SUPPLEMENTAL FIGURES

|                                                                                                                              |    |
|------------------------------------------------------------------------------------------------------------------------------|----|
| Supplemental Figure 2. 1. $^{31}\text{P}$ -NMR proton-decoupled (black) and -coupled (blue) spectra of 10 mM Phi (pH 7)..... | 68 |
| Supplemental Figure 2. 2. PF stability in sterile MYG medium. ....                                                           | 69 |



**Supplemental Figure 2. 1.**  $^{31}\text{P}$ -NMR proton-decoupled (black) and -coupled (blue) spectra of 10 mM Phi (pH 7).



**Supplemental Figure 2. 2.** PF stability in sterile MYG medium. A Proton-decoupled  $^{31}\text{P}$ -NMR spectrum of 100  $\mu\text{M}$  PF in sterile MYG, incubated at 30°C for 4 days. B Quantification of Phi in sterile MYG supplied with PF. The standard addition curve reading was  $-11 \pm 9 \mu\text{M}$  Phi (0 nmol).

## **IV. Phosphite as an Engineered Niche for *Pseudomonas veronii* in a Synthetic Soil Bacterial Community**

### **Abstract**

Bioaugmentation, the process of soil restoration by introducing microorganisms capable of degrading pollutants, is a promising and cost-effective strategy for environmental remediation. A barrier to successful bioaugmentation is the inoculant's failure to establish within the resident microbial community. In an effort to improve inoculant proliferation, we have investigated phosphite as a phosphorus source for selective nutrient supply. Phosphite is an inaccessible form of phosphorus to organisms that lack the capacity for phosphite oxidation to phosphate. We introduced a phosphite dehydrogenase-coding gene (*ptxD*) into the genome of the toluene-degrading bacterium *Pseudomonas veronii* 1YdBTEX2 to couple phosphite metabolism and aromatic hydrocarbon clearance. When inoculated in either soil matrix or liquid soil extract, *P. veronii* proliferates in a phosphite- and toluene-dependent manner in both growing and stable synthetic soil microbial communities. Once toluene is metabolized, *P. veronii* abundance decays and the microbial community recovers diversity and abundance resembling the uninoculated controls. Additional members of the microbial community were also enriched in the presence of phosphite, and genomic analysis suggests these microorganisms utilize an alkaline phosphatase, *phoV*, for phosphite assimilation.

### **Introduction**

Soil contamination poses a considerable threat to human health, ecological health, and food security throughout the world.<sup>1,2</sup> Specifically, aromatic hydrocarbon soil pollutants associated with industrial activity, such as, benzene, toluene, ethylbenzene and *p*-xylene



(BTEx), are acutely toxic and carcinogenic.<sup>3-5</sup> Remediation of contaminated soil can be achieved through the process of bioaugmentation, wherein a microorganism capable of pollutant degradation is inoculated at the affected site. However, this method has variable performance due to poor establishment of the inoculated bacterium into the native soil community and limited nutrient availability.<sup>6-8</sup>

Previous efforts to enhance bioremediation efficiency have focused on phosphorous (P) supplementation, as P is frequently the limiting nutrient for terrestrial microbes.<sup>9</sup> However, inorganic phosphate (Pi), the bioavailable form of P, exhibits poor soil mobility and inefficient uptake, necessitating excessive Pi application which contributes to eutrophication.<sup>10,11</sup> Furthermore, Pi supplementation in P-replete environments would benefit all native and non-native soil microbes, and thus would not provide any specific advantage to the inoculated bioaugmenting microbe. Phosphite (Phi) is an alternative P source that is more mobile in soil, but requires enzymatic oxidation by phosphite dehydrogenases (*ptxD*) or alkaline phosphatases (*pho*). We have previously shown that, when applied to soil, the phosphite oxidizing bacterium *Pseudomonas stutzeri* WM88 is capable of Phi-dependent growth and can oxidize sufficient Phi to support plant growth, which is otherwise not bioavailable and toxic to plants.<sup>12,13</sup> Coupling microbe-selective phosphite oxidation to the utilization of the pollutant as sole carbon and energy source could, in principle, favor specific proliferation of a bioaugmentation inoculant.

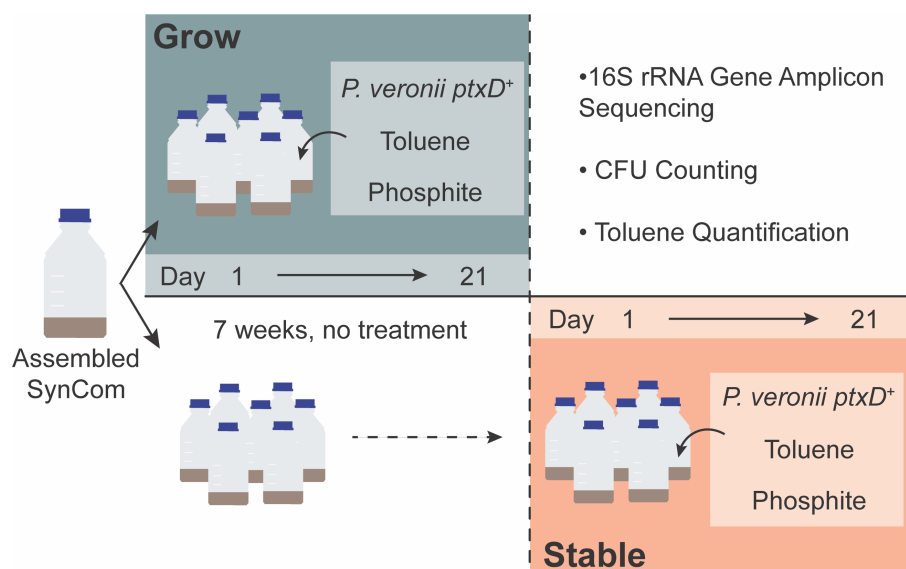
Here, we develop a Phi-dependent bioaugmentation agent by introducing the *ptxD* gene from *P. stutzeri* WM88 into the pollutant degrading bacterium *Pseudomonas veronii* 1YdBTEX2. *P. veronii* 1YdBTEX2 was isolated from jet fuel contaminated soil<sup>14</sup> and can degrade benzene, toluene, ethylbenzene, *m*- and *p*-xylene (BTEx) as carbon sources.

Although the genome of *P. veronii* 1YdBTEX2 contains no genes of any known Phi-oxidizing pathways, it does contain the *phnCDE* operon, known to transport phosphite as well as phosphate and small phosphonates.<sup>15,16</sup> To assess the effect of Phi on the establishment of the *P. veronii* *ptxD*<sup>+</sup> inoculant and toluene degradation rates, we use a model synthetic soil community (SynCom) as our background.<sup>17</sup> This community consists of 21 soil isolates (**Table 2**) covering four major bacterial phyla that have reproducible assembly and

**Table 2.** SynCom members.

| Genus                  | Class               | Phyla          |
|------------------------|---------------------|----------------|
| Microbacterium         | Actinobacteria      | Actinobacteria |
| Mucilaginibacter       | Bacteroidia         | Bacteroidetes  |
| Curtobacterium         | Actinobacteria      | Actinobacteria |
| Variovorax             | Gammaproteobacteria | Proteobacteria |
| Flavobacterium         | Bacteroidia         | Bacteroidetes  |
| Cellulomonas           | Actinobacteria      | Actinobacteria |
| Tardiphaga             | Alphaproteobacteria | Proteobacteria |
| Devosia                | Alphaproteobacteria | Proteobacteria |
| Mesorhizobium          | Alphaproteobacteria | Proteobacteria |
| Burkholderia           | Betaproteobacteria  | Proteobacteria |
| <i>Pseudomonas</i> (1) | Gammaproteobacteria | Proteobacteria |
| Luteibacter            | Gammaproteobacteria | Proteobacteria |
| Chitinophaga           | Bacteroidia         | Bacteroidetes  |
| Lysobacter             | Gammaproteobacteria | Proteobacteria |
| <i>Pseudomonas</i> (2) | Gammaproteobacteria | Proteobacteria |
| Rhodococcus            | Actinobacteria      | Actinobacteria |
| Caulobacter            | Alphaproteobacteria | Proteobacteria |
| Cohnella               | Bacilli             | Firmicutes     |
| Rahnella               | Gammaproteobacteria | Proteobacteria |
| Phenylobacterium       | Alphaproteobacteria | Proteobacteria |
| Bradyrhizobium         | Alphaproteobacteria | Proteobacteria |

successional patterns. In both assembling (“grow”) and established (“stable”) SynComs (**Scheme 6**), Phi enhances *P. veronii ptxD*<sup>+</sup> abundance in toluene-contaminated soil and soil extract, and enhances toluene degradation in early stationary phase. Nevertheless, the coupling of Phi oxidation to toluene degradation is not perfect, and alternative Phi-assimilating pathways present in the SynCom are suggested to explain the loose coupling.



**Scheme 6.** Overview of soil microcosm experimental layout. Two types of resident communities were produced: grow and stable. The grow phase consists of transferring mature SynCom in soil microcosm to fresh soil matrix and soil extract and adding or withholding all combinations of the inoculant *P. veronii ptxD*<sup>+</sup>, toluene in HMN, and Phi. For stable phase, the mature SynCom was transferred to fresh soil matrix and soil extract but was left to develop for 7 weeks without treatment. The previously mentioned conditions were then applied to the stable phase bottles. Timepoints for sampling in each phase are shown on the horizontal axis. On the top right, the sampling methods are outlined.

## Materials and Methods

### Construction of *P. veronii ptxD*<sup>+</sup>

The mini-Tn7 plasmid containing the *ptxD* insertion was constructed using the ClonExpress II One Step Cloning Kit (Vazyme). The pUC18-Tn7-Gm-Amp<sup>18</sup> backbone was linearized by digestion with 40 units of HindIII-HF and SpeI-HF and 1 µg of DNA incubated

at 37°C overnight. The resulting digestion product was isolated and concentrated using a PCR clean-up kit (Macherey-Nagel). The insertion consisted of the promoter region for the phosphate ABC transporter found in *P. veronii* 1YdBTEX2 followed by a ribosome binding site and the *ptxD* sequence of *P. stutzeri* WM88 (Promoter region provided in **Table 3**)<sup>19</sup>. The insertion was amplified by PCR (Primers provided in **Table 3**) using Q5 Hot Start High-Fidelity DNA polymerase (New England Biolabs) from a delivery vector and purified from the agarose gel using a gel extraction kit (Macherey-Nagel). The overlapping ends of the fragments were joined using the ClonExpress kit according to the manufacturer directions.

After the recombination reaction was complete, the DNA product was transformed into *E. coli*

**Table 3.** Primers and DNA sequences used in this study.

| Name               | Sequence                                                                                                                                                                                                                                                                                                                                                                                       | Purpose                                                                                                                                             |
|--------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------|
| P(PstS)            | 5'-<br>GGTGCGGCGGCCTGGGCTTTGCCAGAGGCC<br>ATCACTCCCCTTTCTGATCGTCTGTCAGGAG<br>CGGGCAAGGCTCAGACTTTGCCCCCTCAGC<br>CTCTCCCCCAAAAAACAAAGGGCGAAACAA<br>GGCTAGACCACCTAATGCACAACGGCCAGG<br>CCCTAGCGCCTGGCGCGGTTGGCGCGACCC<br>GCCATTCAACTTTCATAAAAGCTTAACGAAA<br>GTGCTGCAGAGTTCGGGAGCCCGTTATCACA<br>ATGAGTTCCTTCGATGAAAAATTTGATGAAG<br>TCTGCTGCACTCGCCGTTGCGGTTTCTCTTT<br>GTGCAAGTTCCATGGGGAGGACTACTG-3' | Promoter region upstream of <i>ptxD</i> in pUC18-Tn7-Gm-Amp- <i>ptxD</i> , originally the promoter region for <i>P. veronii</i> 1YdBTEX2 PstS gene. |
| <i>ptxD</i><br>Fwd | 5'-ATTCGATCATGCATGAGCTCACT-3'                                                                                                                                                                                                                                                                                                                                                                  | Insertion of P(PstS)-PTDH fragment into pUC18-Tn7-Gm-Amp vector.                                                                                    |
| <i>ptxD</i><br>Rev | 5'-GAGGTACCGGGCCCAA-3'                                                                                                                                                                                                                                                                                                                                                                         |                                                                                                                                                     |
| V3/V4<br>Fwd       | 5'-<br>TCGTCGGCAGCGTCAGATGTGTATAAGAGAC<br>AGCCTACGGGNGGCWGCAG-3'                                                                                                                                                                                                                                                                                                                               | Amplification of 16S V3/V4 region for 16S rRNA gene amplicon sequencing.                                                                            |
| V3/V4<br>Rev       | 5'-<br>GTCTCGTGGGCTCGGAGATGTGTATAAGAGA<br>CAGGACTACHVGGGTATCTAATCC-3'                                                                                                                                                                                                                                                                                                                          |                                                                                                                                                     |

DH5 $\alpha$  cells and the resulting pUC18-Tn7-Gm-Amp-PTDH product was verified by sequencing.

The mini-Tn7 insertion of *ptxD* into *P. veronii* 1YdBTEX2 was initiated by four-parental conjugation. Overnight cultures of *P. veronii* 1YdBTEX2 were prepared in LB (30°C), *E. coli* SM10( $\lambda$ pir) containing the pUX-BF13 Tn7 helper plasmid in LB with 100  $\mu$ g/mL ampicillin (37°C), *E. coli* HB101 containing the pRK2013 conjugation helper plasmid in LB with 25  $\mu$ g/mL kanamycin (37°C), and *E. coli* DH5 $\alpha$  containing the pUC18-Tn7-Gm-Amp-PTDH plasmid in LB with 100  $\mu$ g/mL ampicillin (37°C). Of the 5 mL of each overnight culture, 1.5 mL was spun down at 4,025  $\times$  g for 4 min. The supernatant was removed and the cells were resuspended in 3 mL of fresh LB and grown at 30°C for 2 hours. After incubation, 2 mL of each of the cultures were spun down at 4,025  $\times$  g for 4 min, and the supernatant was removed and cells were resuspended in 100  $\mu$ L LB. Meanwhile, 0.22  $\mu$ m PVDF membrane filters (Sigma-Aldrich) were sterilized using UV light exposure for 2 minutes on each side and placed on LB plates. For conjugation, 50  $\mu$ L of cells were placed on the filters in the following combinations: (1) *P. veronii* 1YdBTEX2, *E. coli* with pUX-BF13 Tn7 helper plasmid, pRK2013 conjugation helper plasmid, and pUC18-Tn7-Gm-Amp-PTDH plasmid; (2) *P. veronii* 1YdBTEX2; and (3) and *E. coli* with pUX-BF13 Tn7 helper plasmid, pRK2013 conjugation helper plasmid, and pUC18-Tn7-Gm-Amp-PTDH plasmid, and incubated at 30°C for 24 hours. Filters were then removed and placed in a 2 mL Eppendorf tube, to which 2 mL of sterile, 0.9% NaCl solution was added. Tubes were vortexed vigorously for 10–15 seconds and 100  $\mu$ L aliquots were placed on M9 minimal media plates supplemented with 20  $\mu$ g/mL gentamicin<sup>20</sup> plates without a carbon source. Plates were placed in an ~4 L plastic sealed container with a 2 mL toluene reservoir for vapor equilibration (toluene chamber),

sealed, and grown at 30°C for 48 hours. No growth was detected from the control plates containing condition (2) nor (3). The *P. veronii* 1YdBTEX2 mutant isolates were re-streaked onto fresh M9 plates with 20 µg/mL gentamicin and toluene to isolate *P. veronii* colonies. Mutant *P. veronii* colonies were verified with colony PCR for the insertion of the mini-transposed fragment using Q5 High-Fidelity 2X Master Mix (New England Biolabs).

### **Soil Microcosm Preparation and Inoculation**

Soil microcosms were prepared as previously described with minor modifications noted below.<sup>17</sup> Soil matrix (SM) was made from riverbank sediment by the Sorge river in Lausanne, Switzerland (46°31'22.4"N 6°34'31.7"E). The sediment was dried and double-sieved to obtain 0.5-3 mm sized particulates. The SM was autoclaved, and a week later was divided into 85 g portions in 500 mL Schott glass bottles (90 g for the initial SynCom inoculation). The individual flasks were closed and autoclaved twice to ensure all spores were killed, and a bottle was tested for sterility by adding 10 g of SM to 10 mL sterile 0.2% (w/v) tetrasodium-pyrophosphate decahydrate (pH 7.5, Sigma Aldrich), vortexing for 1 min, and spreading 100 µL of the supernatant onto a Reasoner's 2A (R2A) agar plate. Soil extract (SE) was collected from the Dorigny Forest in Lausanne, Switzerland (46°31'16.4"N 6°34'43.0"E). Top soil was collected and mixed in a 1:1 ratio with tap water. The mixture was autoclaved and left overnight, then decanted into centrifuge tubes and centrifuged at 5000 × g for 15 min. The supernatant was combined, mixed, and pooled into 1 L Schott bottles before autoclaving and testing for sterility by plating 100 µL onto an R2A agar plate.

The SynCom was assembled from plates of the 21 isolates as described previously.<sup>17</sup> The original SynCom soil microcosms were initiated by inoculating 90 g of SM with 10 mL

of SE containing  $10^7$  cells  $\text{mL}^{-1}$  (total) of the SynCom community, with each member in equal proportion from pure starter cultures, as measured by  $\text{OD}_{600}$ . After inoculation, the bottles were mixed on a horizontal roller mixer for 20 min at 80 rpm and incubated at room temperature in the dark for two months with periodic rotation. A sufficient quantity of this mature SynCom soil microcosm was prepared to yield a combined 400 g and was mixed with a sterile beaker and stirrer prior to use in inoculating experimental microcosms. To inoculate an individual microcosm, 6 g of the combined mature SynCom was added to 85 g of sterile SM, along with 10 mL of sterile SE. Soil microcosms were either used for experiments directly (“grow”) or were mixed on a horizontal roller mixer (20 min at 80 rpm) and incubated at room temperature in the dark for 7 weeks with periodic rotation before experimental conditions were applied (“stable”).

For experiments done exclusively in SE,  $10^7$  cells  $\text{mL}^{-1}$  of the same equal abundance SynCom community used for soil matrix was added to 10 mL of sterile SE (adjusted to pH 8.6) in a 50 mL Falcon tube and vortexed to mix. Falcon tubes were then placed in a room temperature incubator at 100 rpm and incubated for 2 weeks before a 1:10 dilution of mature SynCom into fresh sterile SE.

### **Soil Microcosm Conditions and Sampling**

The soil microcosms were prepared for two experiment phases: “grow” and “stable” (**Scheme 6**). The growing phase exposed SynCom to all permutations of the following treatments: the inoculation of *P. veronii ptxD<sup>+</sup>*, the introduction of toluene, and the addition of Phi, directly after inoculation of fresh SM and SE. Stable phase similarly involved transferring the SynCom to fresh SM and SE, but instead included incubating these bottles in

the dark at room temperature for 7 weeks before exposing the SynCom cultures to the experimental conditions and *P. veronii ptxD*<sup>+</sup> inoculation.

To inoculate *P. veronii ptxD*<sup>+</sup>, the mutant was grown on M9 plates with 20 µg/mL gentamicin and toluene as the carbon source in a toluene chamber. Individual colonies were then picked, inoculated in 10 mL of liquid M9 medium with 20 µg/mL gentamicin and 15 mM succinate and grown overnight at 30°C while shaking at 180 rpm. Prior to inoculation in the soil microcosms, the entire culture was spun down at  $4,025 \times g$  for 4 min and washed 3× with sterile 0.9% NaCl. The cell density was measured using absorbance at 620 nm, and the culture was appropriately diluted to allow for the addition of  $10^5$  cells g<sup>-1</sup> SM in 100 µL of *P. veronii ptxD*<sup>+</sup>.

Toluene (Honeywell), used for simulating contamination in soil matrix, was prepared in a 7.5% v/v solution with 2,2',4,4',6,8,8'-heptamethylnonane (HMN, Sigma Aldrich), and the SE was amended with 2% v/v of the toluene solution and vortexed briefly before addition to the SM. Phi was added directly to the SE to a final concentration of 1.3 mM from a 1.3 M Phi stock (pH 5, Sigma Aldrich) and was filter sterilized prior to soil matrix inoculum preparation. Once the bottles were inoculated with SynCom and all perturbations of the soil experimental condition were applied, the bottles were rolled to mix for 20 min. Control bottles containing only *P. veronii ptxD*<sup>+</sup> or sterile bottles, were subjected to the same conditions. For the SE liquid culture experiments, toluene was introduced in a 35% v/v solution with HMN, and Phi was introduced directly to the SE. After applying these conditions, SE microcosms were vortexed for 30 s.

Timepoints for the growing phase were taken 24 h, 48 h, 4 days, 7 days, and 3 weeks after inoculation. Timepoints for the stable phase were taken 24 h, 48 h, 5 days, 8 days (except



for the Syncom-only control), and 3 weeks. Timepoint measurements consisted of colony forming unit (CFU) counting, the collection of cells for later DNA extraction and 16S rRNA gene amplicon sequencing, and the collection of soil microcosm samples for toluene quantification. Firstly, a sterile inoculation loop was used to collect 100 mg of SM + SE in a 1 mL crimp vial and was immediately stored at  $-80^{\circ}\text{C}$  for toluene analysis. Then, using a Sterileware spatula (SP Bel-Art), 10 g of SM + SE was removed from the bottle and placed in a 50 mL sterile Falcon tube with 10 mL of 0.2% pyrophosphate solution. The tube was vortexed at maximum speed for 2 min, then the particles were allowed to settle for 1 min. The top layer ( $\sim 5$  mL) of the suspension was removed and placed in a 15 mL sterile Falcon tube. From this, a 100  $\mu\text{L}$  aliquot was removed, subjected to several 1:10 serial dilutions, and plated on R2A agar plates for CFU counting. The remaining cell suspension was spun down at  $4,025 \times g$  for 4 min, then the supernatant was removed and the cells were resuspended in  $\sim 0.5$ –1 mL of the pyrophosphate solution and placed in a new 1.5 mL Eppendorf tube. The cells were spun down once more, and the supernatant was removed. Tubes containing the cell pellet were frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  for subsequent DNA isolation (see below).

For the SE liquid culture experiments, CFU counts, genetic diversity, and toluene were also measured. Prior to sampling, the culture vials were vortexed at maximum speed for 30 s. Toluene samples were taken as 10  $\mu\text{L}$  SE culture and were placed in crimp vials and stored at  $-80^{\circ}\text{C}$ . A 100  $\mu\text{L}$  aliquot was taken directly from the culture for CFU counting as described above. Finally, 1 mL of culture was spun down at  $6000 \times g$  for 4 min, the supernatant was removed, and the cell pellet was snap frozen with liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until DNA isolation.

## Community 16S rRNA Gene Amplicon Sequencing

DNA was purified from thawed cell pellets using the DNeasy PowerSoil Pro kit (Qiagen) according to the manufacturer's instructions. Purified DNA concentrations were quantified using the Qubit dsDNA BR assay kit (ThermoFischer Scientific), diluted to 10 ng/ $\mu$ L, and stored at -20°C. Library preparations were performed according to the Illumina 16S Metagenomic Sequencing Library protocol ([https://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry\\_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf](https://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf)) for the V3 to V4 region of the 16S rRNA gene (Primers provided in **Table 3**). PCR clean-up was performed using CleanNGS beads (CleanNA, The Netherlands). The gene library was indexed using Nextera XT Index Kit Set A and B (v2, Illumina Inc., USA). Libraries were supplied with 40% PhiX control, and sequenced using 300 cycle paired-end sequencing on MiSeq v3 (Illumina Inc., USA) for SM experiments and Aviti (Element Biosciences, USA) for liquid SE experiments at the Lausanne Genomic Technologies Facility.

The raw sequencing reads were assessed for quality using FastQC 0.11.9<sup>21</sup>, then trimmed and paired using Trimmomatic 0.39<sup>22</sup> with a sliding window of 5:28. The paired reads were merged using Flash 1.2.11<sup>23</sup> with a minimum and maximum overlap of 20 bp and 170 bp, respectively. To tally abundances of SynCom and inoculant strains, merged sequences were queried using the Bash “grep” command for regions of V3/V4 that were unique to each strain, as determined previously<sup>17</sup>. Strain relative abundances were determined by first normalizing counts for each organism to the number of 16S rRNA gene operons found in the

given strain then normalizing to total counts detected. Relative abundances were then combined with the CFU counting data to determine absolute abundances of the present strains.

### **Toluene Quantification by GC-MS**

Toluene analysis was performed on an Agilent 8890 GC System (GC, Agilent Technologies Inc., USA) paired to an Agilent Series 5977B mass selective detector (MS). Prior to analysis, samples were removed and heated to 26°C in a dry bath for 10 minutes prior to incubation with a solid phase micro-extraction (SPME) fiber. The SPME fiber (Car-WR/PDMS, Agilent Technologies, Inc.) was conditioned prior to sampling runs by exposing the fiber to 295°C for 30 minutes (Split injection, 1:30) inside the GC inlet. For sample collection, a small gauge needle was used to puncture a hole in the 1 mL crimp vial cap and the fiber needle was inserted ~1 cm into the vial headspace and exposed for 2 minutes before injecting into the GC-MS. The gas sample was resolved on a HP-5MS Ultra inert (Agilent Technologies, Inc.) column. Acquisition parameters for GC-MS analysis were as follows: inlet temperature was 275°C and run in splitless mode; oven heated to initial temperature of 39°C and held for 4 min, then increased 15°C/min to 90°C, then increased 30°C/min to 275°C and was held for 1.43 min; transfer line temperature was 275°C. Quantification was done using MassHunter Quantitative Analysis Software (Agilent Technologies, Inc.). For the external calibration curve, 0–7.5% toluene (v/v) in HMN was amended to SE and SM at 2% v/v.

### **Statistical Analyses**

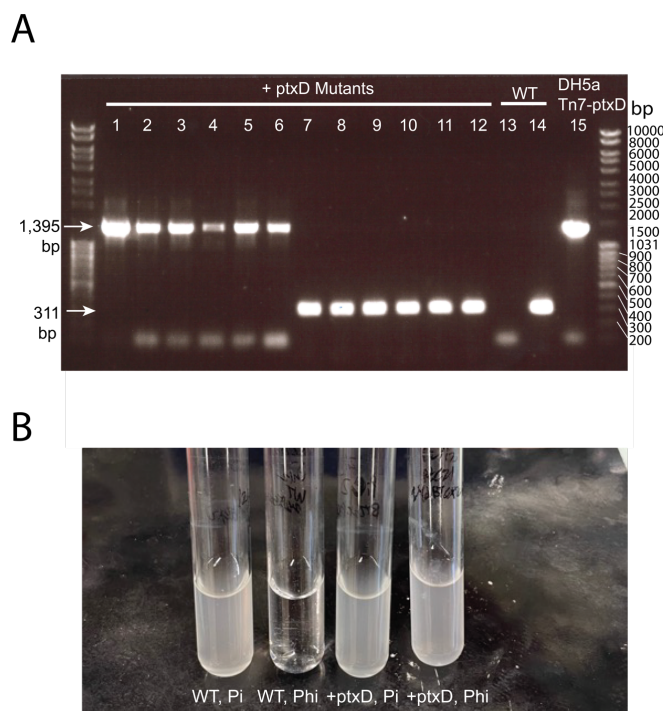
All data analysis was performed in R (R Core Team, 2023) with packages *vegan*<sup>24</sup>, *ggplot2*<sup>25</sup>, and *dplyr*<sup>26</sup>. Community composition across conditions and timepoints was analyzed using strain relative and absolute abundance data and Bray-Curtis distances determined using the *vegdist* function in *vegan* and was then ordinated using non-metric multidimensional scaling (NMDS) with function *metaMDS*. To determine differences between treatments in NMDS plots, Adonis (PERMANOVA with 999 permutations) was used with function *adonis2* in the *vegan* package. Alpha diversity was determined using *diversity* (Shannon and Simpson indices) and *specnumber* (richness) from *vegan*, and differences between conditions were evaluated using calculated p values from the Welch's t-test. Enriched or depleted community members for the applied conditions were determined by inputting the absolute or relative strain abundances into a linear regression model, and outliers were identified as those with a  $>1 \log_{10}$  difference in the actual values to the theoretical values determined in the model<sup>27</sup>. All outliers identified were confirmed as outside the 99% confidence interval for the linear regression model. The effect of time and inoculant presence or absence, or time and Phi presence or absence, on toluene concentrations was evaluated using two-way ANOVA. The effect of time and Phi presence or absence on *P. veronii ptxD*<sup>+</sup> abundance was also assessed using two-way ANOVA.

## Results

### Phi-oxidizing Capacity of *P. veronii ptxD*<sup>+</sup>

Mutant *P. veronii* colonies were verified with colony PCR to confirm the organism species and demonstrate the insertion of the *ptxD* gene (**Figure 28A**). To verify that the *ptxD* insertion conveyed the functional ability to oxidize Phi, 21C liquid media<sup>28</sup> was prepared with Phi replacing Pi as the phosphorus source (23 mM Phi) and buffer system at pH 6.8 and 10

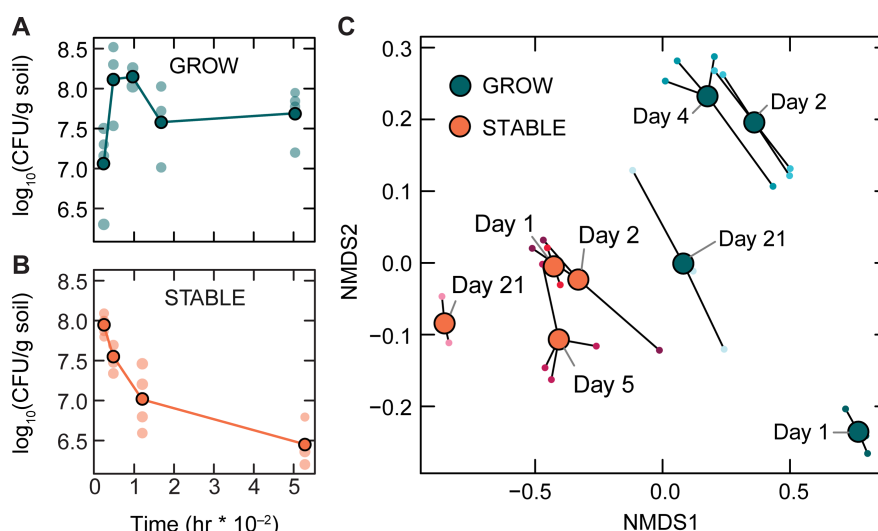
mM succinate as the carbon (C) source. *P. veronii* 1YdBTEX2 WT or *ptxD*<sup>+</sup> mutant colonies were inoculated into 5 mL of Phi- or Pi-containing 21C media. After incubating at 30°C for 2 days, cultures were checked for growth on Phi by their culture turbidity. While both the WT and mutant strain were capable of growth in Pi medium, only *ptxD*<sup>+</sup> mutants displayed growth in the Phi medium (**Figure 28B**).



**Figure 28.** Confirming the insertion and functionality of the *ptxD* gene in *P. veronii* 1YdBTEX2. **A** Agarose gel electrophoretic mobility describing the isolated mutant strain (lanes 1–12). The *ptxD* insertion is shown by the amplification of the 1,395 bp sequence by colony PCR (lanes 1–6). The amplification of the same sequence from a colony of DH5a *E. coli* containing the pUC18-Tn7-Gm-Amp-PTDH plasmid is shown as a positive control (lane 15), and from wild type (WT) *P. veronii* as a negative control (lane 13). A 311 bp sequence on chromosome 2 of *P. veronii* was amplified in the mutant strain by colony PCR to confirm the species identity (lanes 7–12). WT *P. veronii* was amplified using the same sequence primers as a positive control (lane 14). **B** Growth of WT *P. veronii* and the *P. veronii ptxD*<sup>+</sup> mutant on 21C media containing either phosphate (Pi) or phosphite (Phi) as the sole phosphorus source.

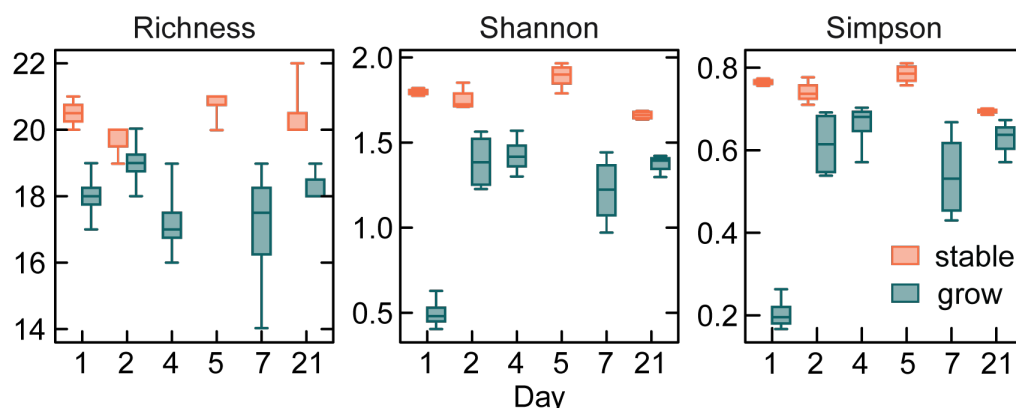
### SynCom Growth and Toluene Response

To evaluate the efficacy of *P. veronii* *ptxD*<sup>+</sup> in enabling Phi-stimulated soil remediation within a microbial consortium background, we first measured the model SynCom’s growth and diversity characteristics in the absence of *P. veronii* or any additional chemical perturbations. In the “grow” stage, the transfer of assembled SynCom to fresh SE and SM allowed the community to reach maximum cell densities between 10<sup>8</sup>–10<sup>9</sup> CFU/g soil between 24 h and 48 h post-inoculation (**Figure 29A**). After 4 days in the grow phase, the community cell viability decreased to 10<sup>7</sup>–10<sup>8</sup> CFU/g soil and remained in this range until the beginning of “stable” phase, 7 weeks after the initial inoculation. During stable phase, cell densities dropped steadily to 10<sup>6</sup>–10<sup>5</sup> CFU/g soil (**Figure 29B**), which is consistent with previous observations<sup>17</sup>. We also measured community composition by 16S rRNA gene amplicon sequencing during both grow and stable phases (**Supplemental Figure 3.1**). After the 24 h, the most abundant SynCom member in the grow phase was *Pseudomonas* sp. strain



**Figure 29.** SynCom development in the soil microcosms. **A** Total colony forming units (CFU) per g soil over time of the SynCom in the grow phase. Average of biological replicates shown in dark aqua and individual measurements in translucent aqua. **B** Total colony forming units (CFU) per g soil over time of the SynCom in the stable phase. Average of biological replicates shown in orange and individual measurements in translucent orange. **C** Non-metric multidimensional scaling analysis of the SynCom species relative abundance data during the grow (dark aqua) and stable (orange) phase of development over time using Bray-Curtis distance values (stress = 0.026). Daily centroids are shown as outlined circles and replicates are shown as smaller circles, colored according to timepoint and community phase.

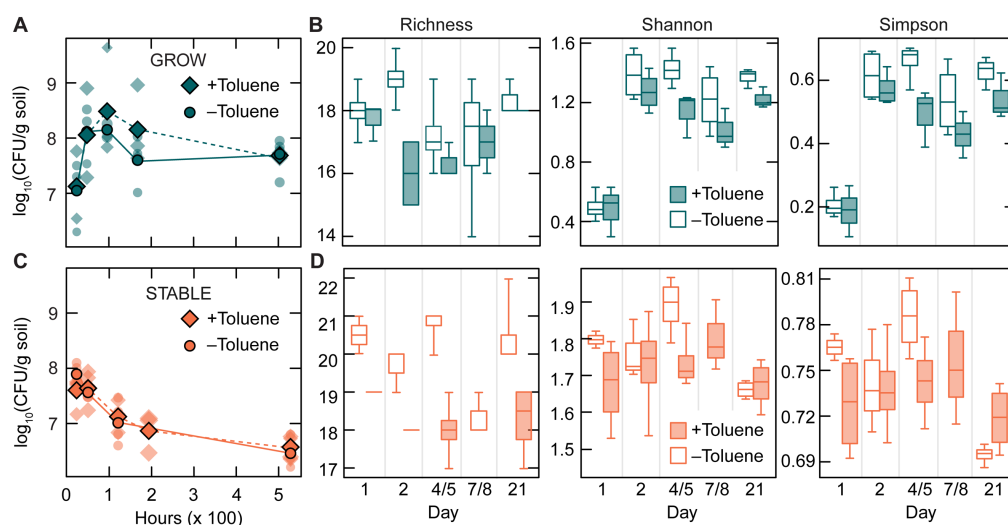
2, but became less prominent with time throughout grow and stable phases, with a concomitant increase of *Lysobacter*, a facultative predatory bacterium. The ultimate decay of the initial *Pseudomonas* sp. strain 2 population over the grow and stable phases was correlated with an increase in alpha diversity (**Figure 30**). The SynCom dynamics over the grow and stable phases were distinctly clustered by non-metric multidimensional scaling (NMDS) ordination of their relative species abundances (**Figure 29C**).



**Figure 30.** Bar plots showing alpha diversity of SynCom in the grow and stable phase. Diversity was determined using three metrics: richness ( $p = 3.425 \times 10^{-7}$ ), Shannon index ( $p = 1.716 \times 10^{-6}$ ), and Simpson index ( $p = 6.984 \times 10^{-5}$ ).

Next, we sought to investigate the SynCom response to a model soil contaminant, toluene. To avoid evaporative losses and to decrease toxicity, toluene was introduced into the soil microcosm as a solute in the inert carrier solvent 2,2',4,4',6,8,8'-heptamethylnonane (HMN)<sup>29</sup>. The total SynCom growth after mixing with fresh soil matrix (grow phase) containing 130  $\mu\text{g}$  toluene per g soil was indistinguishable from growth in soil without toluene (**Figure 31A**), and toluene loads as high 865  $\mu\text{g/g}$  soil could be tolerated as long as HMN was used as the delivery mechanisms (**Supplemental Figure 3.2**). The community composition (**Supplemental Figure 3.3**) and alpha diversity analysis showed a significant change in

richness ( $p = 0.026$ , Welch's t-test), but no change in the Shannon or Simpson diversity indices (**Figure 31B**). Toluene exposure during the stable phase also showed no change in total cell counts over the course of the 21 day observation period (**Figure 31C**) and a decrease in richness ( $p = 4.83 \times 10^{-8}$ , **Figure 31D**). Based on a NMDS ordination and Bray-Curtis distances, there is no statistically significant effect of the addition of 130  $\mu\text{g}$  toluene per g soil on the SynCom development, either in grow or stable phases ( $p = 0.43$  by PERMANOVA, **Supplemental Figure 3.4**).

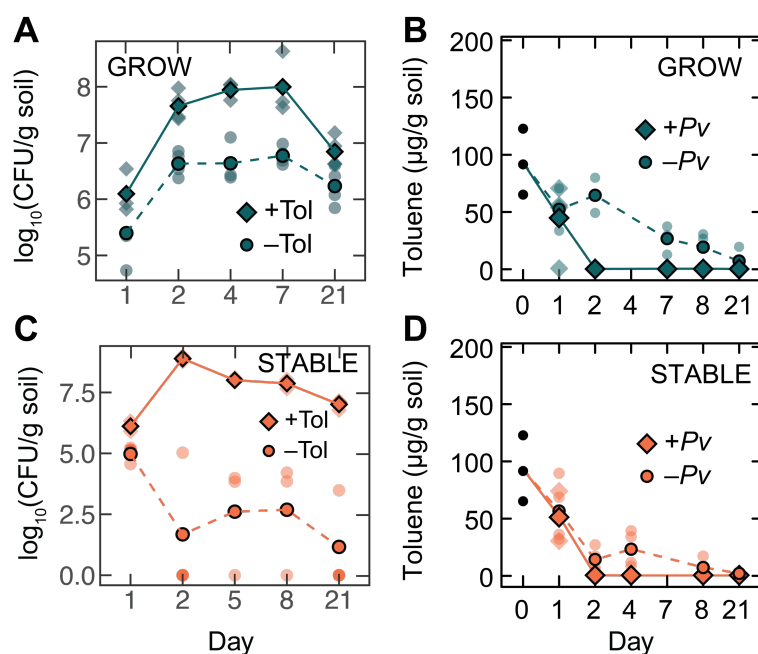


**Figure 31.** Effect of toluene exposure on SynCom. **A** Cell densities of SynCom in grow phase in the presence or absence (reproduced from Figure 1A for comparison) of toluene. Samples collected on days 1, 2, 4, 7, and 21. **B** Bar plots showing alpha diversity of SynCom with (filled boxes) and without (empty boxes) toluene in the grow phase according to three metrics: richness ( $p = 0.02635$ , Welch's t-test), Shannon index ( $p = 0.2895$ , Welch's t-test), and Simpson index ( $p = 0.2223$ , Welch's t-test). **C** Cell densities of SynCom in stable phase 24 h after transfer at varying toluene concentrations. Samples collected on days 1, 2, 5, 8, and 21. **D** Bar plots showing alpha diversity of SynCom with and without toluene in the stable phase according to three metrics: richness ( $p = 4.831 \times 10^{-8}$ , Welch's t-test), Shannon index ( $p = 0.1487$ , Welch's t-test), and Simpson index ( $p = 0.5386$ , Welch's t-test).

### Toluene Removal by *Pseudomonas veronii* ptxD<sup>+</sup>



To evaluate the potential for Phi-dependent niche establishment of a toluene remediating *P. veronii ptxD*<sup>+</sup> bioaugment, we first measured the response of toluene-exposed SynComs to *P. veronii ptxD*<sup>+</sup> inoculation. When applied to a toluene-contaminated SynCom in the grow phase, *P. veronii ptxD*<sup>+</sup> achieved cell densities of 10<sup>7</sup>–10<sup>8</sup> CFU/g soil (**Figure 32A**) and became the dominant SynCom member between 24 h and 48 h and remained so for the entirety of the 21 days (**Supplemental Figure 3.5**). We also measured toluene metabolism and evaporative losses using headspace solid-phase microextraction (HS-SPME)<sup>30,31</sup> and gas chromatography-mass spectrometry. In the absence of any SynCom or bioaugmentation, toluene slowly evaporated from the soil microcosm over the course of 21 days, with >40% of



**Figure 32.** Effect of *P. veronii ptxD*<sup>+</sup> application to toluene-contaminated SynCom. Absolute abundances of *P. veronii ptxD*<sup>+</sup> in toluene-contaminated soil microcosm with (diamonds) and without (circles) toluene in **A** grow ( $p = 3.41 \times 10^{-4}$ , two-way ANOVA) and **C** stable ( $p = 5.85 \times 10^{-10}$ , two-way ANOVA) phase. Toluene concentrations in SM + SE for **B** grow ( $p = 0.0108$ , two-way ANOVA) and **D** stable ( $p = 0.0185$ , two-way ANOVA) phase SynCom with (diamonds) and without (circles) *P. veronii ptxD*<sup>+</sup>. Average of biological replicates for **A–D** shown in dark aqua or dark orange and individual measurements in translucent aqua or orange.

the initial toluene present retained at day 8 (**Supplemental Figure 3.6**). In the presence of *P.*

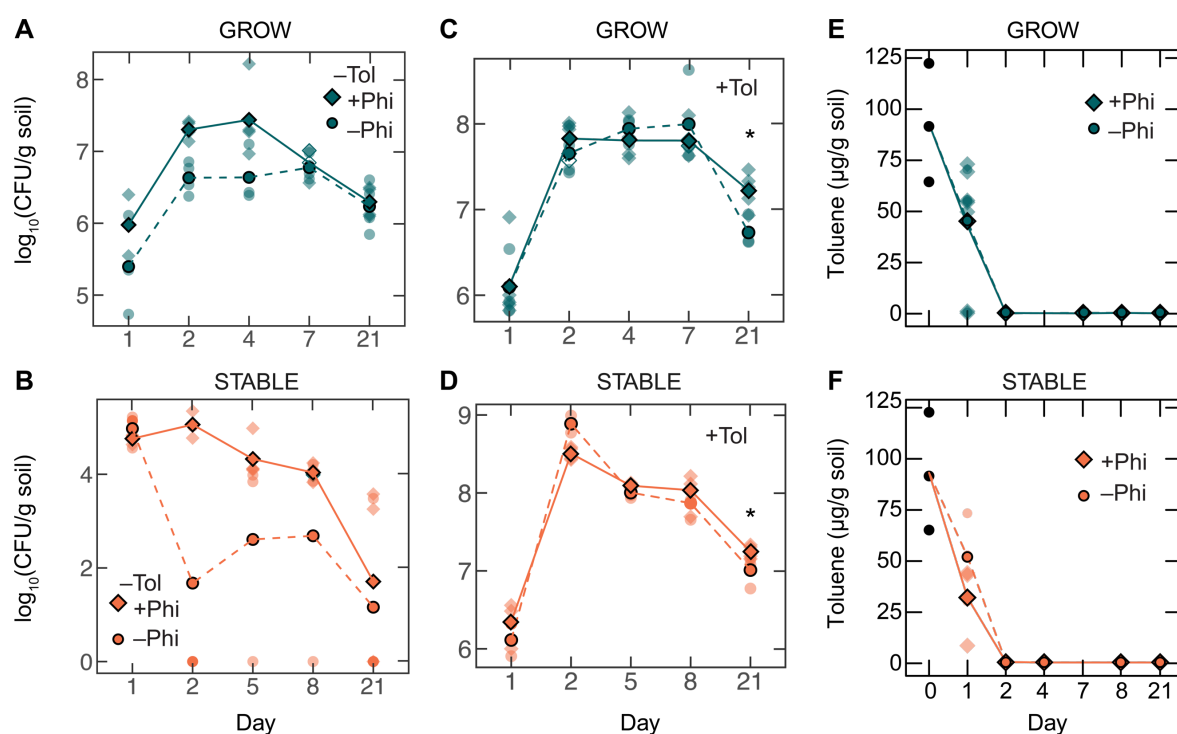
*veronii ptxD*<sup>+</sup>, toluene concentrations decayed significantly faster from the soil microcosm, both in mono-culture (**Supplemental Figure 3.6**,  $p = 0.012$  by two-way ANOVA) and within the SynCom during grow phase (**Figure 32B**,  $p = 0.011$ ), with toluene < 0.5% of the initial concentration after two days. Comparing the net toluene loss to cellular abundance of *P. veronii ptxD*<sup>+</sup> at 48 h yields an apparent toluene carbon consumption rate of  $5.9 \times 10^{-5}$  gC/g<sub>soil</sub>. This carbon consumption is greater than the carbon associated with *P. veronii* biomass growth over the same time period, only  $2.3 \times 10^{-8}$  gC/g<sub>soil</sub>, assuming 280 fg C per cell and a carbon to biomass yield of 10%, suggesting toluene metabolism by *P. veronii* contributes to the growth of other SynCom members, perhaps through cross-feeding, as suspected previously.<sup>27</sup>

The effect of *P. veronii ptxD*<sup>+</sup> inoculation to toluene-contaminated stable phase SynCom soil microcosms were more pronounced. The inoculant reached higher relative abundances in the community during the stable phase (**Figure 32C**), contributing up to 84% of the total community counts compared to 55% maximal population in the grow phase (**Supplemental Figure 3.5**). Toluene loss in the stable phase was also accelerated relative to the rate of evaporative loss (**Figure 32D**,  $p = 0.019$ ) and, similarly to grow phase, <0.6% of initial toluene levels were present after 2 days. Unlike the grow phase, toluene consumption was correlated with *P. veronii* biomass in the stable phase, with a toluene consumption rate of  $1.2 \times 10^{-5}$  gC/g<sub>soil</sub> between 24 h and 48 h, a value near the predicted value of  $4.8 \times 10^{-5}$  gC/g<sub>soil</sub>.

### **The Effect of Phosphite on Toluene Removal**

The application of Phi to toluene-contaminated soil microcosm offers a potential route for inoculant establishment through nutritional niche selectivity. To investigate phosphorus nutritional niche selection of the *P. veronii ptxD*<sup>+</sup> bioaugment, we measured the effect of Phi

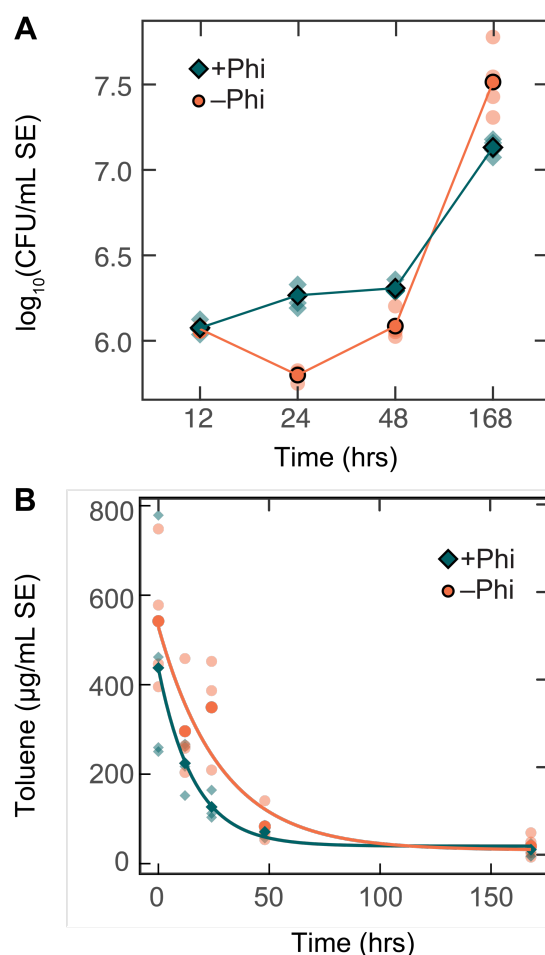
supplementation on growth rates and toluene clearance in soil microcosms. In the absence of the SynCom, *P. veronii* *ptxD*<sup>+</sup> growth and toluene clearance were unaffected by 12 µg Phi per g soil (**Supplemental Figure 3.7**). In contrast, when inoculated with the SynCom *P. veronii* *ptxD*<sup>+</sup> was enriched in the soil microcosm upon the application of Phi (**Figure 33A** and **33B**,  $p = 0.035$  and  $0.090$  by two-way ANOVA for grow and stable phase, respectively). In the presence of toluene, *P. veronii* *ptxD*<sup>+</sup> achieved higher absolute abundances in soil microcosm (**Figure 33C** and **33D**), but applied Phi had no effect on *P. veronii* *ptxD*<sup>+</sup> or any other SynCom



**Figure 33.** Phi effect on toluene-remediation system. Absolute abundances of *P. veronii* *ptxD*<sup>+</sup> in toluene-free soil microcosm with and without Phi in **A** grow ( $p = 0.035$ , two-way ANOVA) and **B** stable ( $p = 0.090$ , two-way ANOVA) phase of SynCom, and toluene-contaminated soil microcosm with and without Phi in **C** grow and **D** stable phase of SynCom. Timepoints of increased *P. veronii* *ptxD*<sup>+</sup> abundance in toluene-contaminated microcosm are highlighted for Day 21 of grow phase ( $p = 0.019$ , Welch's t-test) and Day 21 of stable phase ( $p = 0.057$ , Welch's t-test). Toluene concentrations in SM + SE for **E** grow and **F** stable phase SynCom with *P. veronii* *ptxD*<sup>+</sup> with (diamonds) and without (circles) Phi. Average of biological replicates for **A–F** shown in dark aqua or dark orange and individual measurements in translucent aqua or orange.

member (**Supplemental Figure 3.8**). Only for the Day 21 timepoint for grow phase ( $p = 0.019$ , Welch's t-test) and stable phase ( $p = 0.057$ , Welch's t-test) could a 3-fold (grow) and 2-fold (stable) increase in *P. veronii* *ptxD*<sup>+</sup> abundance be observed. Within the SynCom community, *Pseudomonas* 2 remained the largest competitor for absolute abundance during the grow phase, whereas *Lysobacter* was most competitive during the stable phase (**Supplemental Figure 3.8**). The similarity of the community composition along the grow and stable phase is shown in the **Supplemental Figure 3.9** NMDS plot ( $p = 0.79$  by PERMANOVA). No Phi effect was observed for toluene clearance, which was complete within the first 48 h in both grow and stable phases with or without Phi. The data are consistent with toluene serving a significant role in stimulating *P. veronii* *ptxD*<sup>+</sup> growth, and Pi in the soil microcosm being insufficiently depleted to necessitate Phi metabolism, except when competing among the SynCom for soil microcosm carbon.

To examine how nutrient availability affects toluene clearance in a bioaugmented environment we used liquid soil extract (SE), which has lower Pi availability and suffers less from evaporative loss of toluene. The SynCom composition was distinct in the SE at all time points over the 21-day incubation, relative to the soil matrix microcosm in either grow or stable phase, with a much larger relative abundance of *Rahnella* and *Pseudomonas* 1 (**Supplemental Figure 3.10**). We also increased the toluene concentration from 130  $\mu\text{g}$  toluene/g soil to 600  $\mu\text{g}$  toluene/mL to ensure abundant substrate and enhance a potential advantage for Phi on *P. veronii* growth (assuming Pi-limitation would show more rapidly at higher net *P. veronii* growth). Indeed, *P. veronii* *ptxD*<sup>+</sup> exhibited higher absolute abundances in toluene-contaminated SE medium in the presence of Phi for the first 48 h after inoculation than in its absence (**Figure 34A**,  $p = 0.00025$ , two-way ANOVA). This 3-fold increase of *P.*



**Figure 34.** The Phi effect in liquid SE. **A** Absolute abundances of *P. veronii ptxD*<sup>+</sup> in toluene-contaminated, SynCom-containing SE with (dark aqua diamonds) and without Phi (orange circles) ( $p = 0.00025$ , two-way ANOVA). Outlined points represent the average value of biological replicates. **B** Toluene concentrations with (dark aqua diamonds) and without (orange circles) Phi measured in toluene-contaminated SE containing SynCom and *P. veronii ptxD*<sup>+</sup>. Outlined points represent the average value of biological replicates. An exponential fit of the data is shown as a solid lines with kinetic constants  $-0.04 \pm 0.01 \text{ h}^{-1}$  in the absence of Phi and  $-0.06 \pm 0.03 \text{ h}^{-1}$  in the presence of Phi.

*veronii ptxD*<sup>+</sup> abundance at 24 h coincided with nearly 3-fold lower toluene concentrations in the Phi-amended SE cultures at the 24 h timepoint (**Figure 34B**,  $p = 0.050$ , Kruskal-Wallis test). Using an exponential fit of the data, toluene consumption was  $-0.06 \pm 0.03 \text{ h}^{-1}$  in the presence of Phi and  $-0.04 \pm 0.01 \text{ h}^{-1}$  in the absence of Phi. Toluene-exposed and inoculant-induced SynCom cultures demonstrated a drop in cell viability at the 24 h timepoint that was

completely recovered in the cultures supplemented with Phi (**Supplemental Figure 3.11**,  $p = 0.018$ , Kruskal-Wallis test).

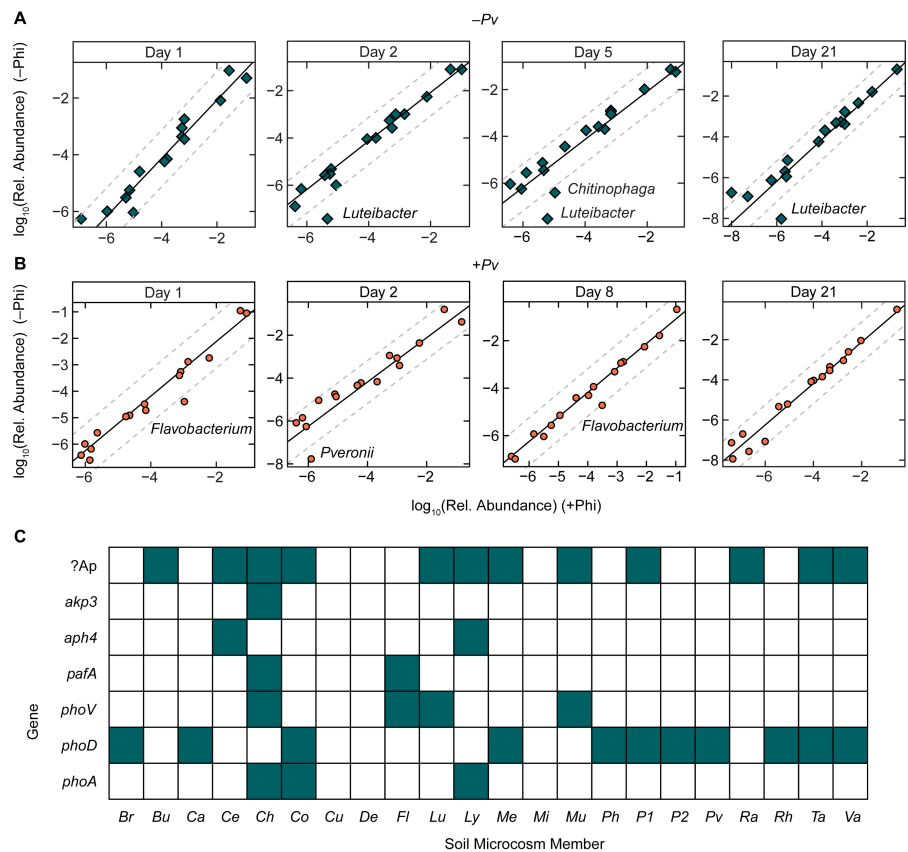
### Phi Oxidation by SynCom Members

Despite improved establishment of *P. veronii* *ptxD*<sup>+</sup> in Phi-supplemented soil microcosm and faster toluene clearance, alternative Phi-metabolism pathways in SynCom members could compromise the Phi-dependent bioaugment stimulation. We examined the SynCom member's annotated genomes for homologs to known Phi-oxidizing pathways, encoded in the *ptx*, *htx*, and *phn* operons<sup>32,33</sup>, using the NCBI blastp algorithm (E value cut-off of  $1 \times 10^{-50}$ , **Table 4**). Sequence alignments of *ptxD* with glyoxylate/hydroxypyruvate reductase B found in *Burkholderia* and *Variovorax* show similar percent sequence identities as other NAD<sup>+</sup>-dependent D-hydroxy acid dehydrogenases of known function have with *ptxD*.<sup>34</sup> Another pathway for Phi-oxidation in *Escherichia coli* is the bacterial alkaline phosphatase (APase) *phoA*, which catalyzes Phi disproportionation to phosphate and molecular hydrogen.<sup>35</sup> While APases primarily act on phosphomonoesters, their substrate promiscuity is well-established and thought to play an important role in the biogeochemical P

**Table 4.** pBLAST hits for *ptxD* in SynCom members' annotated genome with E-values  $< 1 \times 10^{-50}$ .

| SynCom Member | Description                                    | E-value              | % Identity | % Query Cover |
|---------------|------------------------------------------------|----------------------|------------|---------------|
| Burkholderia  | Glyoxylate/hydroxypyruvate reductase B         | $4 \times 10^{-54}$  | 34.81      | 93            |
| Cohnella      | Chromosomal replication initiator protein DnaA | $9 \times 10^{-139}$ | 51.61      | 76            |
| Variovorax    | Glyoxylate/hydroxypyruvate reductase B         | $4 \times 10^{-62}$  | 33.23      | 97            |

cycle.<sup>36–38</sup> We compared SynCom relative abundances in soil microcosms with or without addition of Phi (**Figure 35A, Supplemental Figure 3.12**) and in the presence of *P. veronii* *ptxD*<sup>+</sup> (**Figure 35B**) to identify any enriched SynCom members in soil microcosms. Community analysis showed elevated relative abundance of *Chitinophaga* and *Luteibacter* in SynCom supplemented with Phi in stable phase (**Figure 35A**). In the presence of *P. veronii*, *Flavobacterium* was enriched in the soil microcosm (**Figure 35B**). Of the APases identified



**Figure 35.** The Phi effect on SynCom development and alkaline phosphatase gene presence in SynCom members. **A** Linear regression model comparing strain relative abundance data for SynCom in soil microcosm with (x-ordinate) versus without (y-ordinate) Phi application. Enriched strains, those that deviate more than 1 log away (dashed lines) from the linear model (solid line), are highlighted by their strain name. **B** Linear regression model comparing strain abundance data for SynCom and *P. veronii* *ptxD*<sup>+</sup> in soil microcosm with or without Phi application. **C** Alkaline phosphatase genes present in the SynCom member annotated genomes as well as *P. veronii* *ptxD*<sup>+</sup>. The SynCom members (and *P. veronii* *ptxD*<sup>+</sup>) are all represented by the first two letters of their genus, except for *Pseudomonas* sp. CFSAN084952 (P1), *Pseudomonas moraviensis* (P2).

in the annotated genomes of the SynCom members, *phoV* was the only one shared by *Flavobacterium*, *Chitinophaga*, and *Luteibacter* (**Figure 35C**). Although *phoV* was also identified in the *Mucilaginibacter* genome, this member did not reach abundances above the detection limit for any soil microcosm condition. The protein encoded by the *phoV* gene has been investigated in the cyanobacterium *Synechococcus elongatus* and was shown to be a Pi-irrepressible alkaline phosphatase, the same enzyme family of the widely-distributed and Pi-independent phosphatase *pafA*.<sup>37,39,40</sup> To determine if these strains were capable of Phi oxidation, individual members were inoculated in 5 mL 21C liquid media prepared with Phi replacing Pi as the phosphorus source (23 mM Phi) and buffer system at pH 6.8 and 10 mM succinate as the carbon (C) source. Of these three enriched strains, only *Chitinophaga* and *Luteibacter* were capable of growth on Phi.

## Discussion

In this study, we have assessed the effectiveness of Phi as a nutrient for selectively promoting the growth of *P. veronii ptxD<sup>+</sup>*, a genetically engineered Phi-oxidizing strain of the toluene-degrading soil bacterium *P. veronii* 1YdBTEX2. Our results demonstrate that the presence of Phi leads to increased abundance of the inoculant and faster toluene degradation rates, with the extent of enhancement depending on the growth stage of the resident microbial community and the availability of Pi. The lessened response of the *P. veronii ptxD<sup>+</sup>* inoculant to Phi in comparison to the response to toluene suggests that the soil microcosm model used in this study is C-limited, rather than P-limited, which may not be representative of potential contamination sites.<sup>41,42</sup> This reduced response to Phi is particularly evident in early growth phase, when Pi availability is highest upon transfer to fresh substrate, and no Phi selection is observed until Day 21. Conversely, Phi appears to be most effective in promoting the



establishment of the inoculant and increasing toluene consumption rates during early stable phase of the SM + SE experiments and during the SE liquid experiments, where background Pi concentrations are lower due to P assimilation into biomass and low Pi solubility in water, respectively. Although Pi does not act as an enzymatic inhibitor to PtxD<sup>19</sup>, Phi will likely not supply competitive advantage at sufficiently high Pi concentrations. Therefore, the efficacy of a Phi-directed remediation system is highly contingent on the environmental context. This system might be most appropriately applied to soil sites with low bioavailable P, an increasing proportion of the global terrestrial biosphere.<sup>43,44</sup>

This study provides additional insight into the establishment of an inoculant into a soil microbiome through the quantification of toluene concentrations with HS-SPME. Notably, the observed net toluene consumption in grow phase does not correspond to equivalent growth in *P. veronii* ptxD<sup>+</sup> biomass. This could be due to metabolite leakage commonly found in microbial communities as part of mutually-beneficial process of cross-feeding.<sup>45,46</sup> Cross-feeding of toluene metabolites has been shown previously in toluene-contaminated, naturally-derived soil communities inoculated with *P. veronii* 1YdBTEX2.<sup>27</sup> A similar effect may be present in relation to Phi-oxidation by *P. veronii* ptxD<sup>+</sup>, through the transformation and “leakage” of Phi to broadly available Pi, facilitating growth of other SynCom members and aggravating the resource competition from the perspective of the inoculant.

In addition, we showed that there may be a greater potential for Phi oxidation in soil communities than previously thought. The enrichment of three SynCom members in the presence of Phi suggests that Phi assimilation is more widely distributed in soil communities than the 1.5% of sequenced bacteria and archaea predicted to assimilate Phi based on the prevalence of ptxD.<sup>47</sup> Given the increasing interest in the use of Phi as a selective fertilizer for

transgenic plants, the Phi-oxidizing capabilities of native soil communities should be considered, especially in Pi-abundant environments.<sup>11,48,49</sup> Phi-oxidizing alkaline phosphatases have only recently been proposed as an alternative to *ptxD* for Phi-selection in transgenic plants. The performance of Phi-oxidizing alkaline phosphatase selection systems in soil communities in comparison with *ptxD*-based selection systems, and the impact of hydrogen generation on neighboring microbial metabolism, merits further study.

## **Conclusion**

The application of a P-selective BTEX-remediating strain, *P. veronii ptxD*<sup>+</sup>, increased the efficiency of toluene degradation in soil matrix and liquid environments. The application of Phi serves as a (somewhat) selective and transient nutritional niche in this soil remediation system that allows for increased proliferation of the inoculant in certain environmental and biological contexts. Phi supplementation to SynCom revealed three enriched strains, despite the absence of canonical Phi-oxidation pathways. This suggests that the poorly understood Phi-oxidizing and H<sub>2</sub>-evolving activity of alkaline phosphatase might play a larger role in the biogeochemical redox P cycle of the terrestrial biosphere.

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support in the operation and interpretation of the GC-MS data. This work was supported by the Swiss National Centre for Competence Research *NCCR Microbiomes* (grant number 180575 to JM). CAB gratefully acknowledges financial support for this work from the Fulbright U.S. Student Program, which is sponsored by the U.S. Department of State and the Swiss Federal Commission for Scholarships (FCS). Its contents are solely the responsibility of the author and do not necessarily represent the official views of the Fulbright Program, the Government of the United States, or the FCS. We gratefully acknowledge Prof. Wifred van der Donk for providing the pET-15b 17X-PTDH plasmid. B. L. G. acknowledges a UCSB Faculty Research Grant and the Reagents Junior Faculty Fellowship for financial support.

### **Data Availability Statement**

The raw reads of the 16S rRNA gene V3-V4 amplicon sequencing for the communities in this study are available from the Short Read Archive under BioProject Number PRJNA1136878 for soil microcosms experiments (SM + SE) and BioProject Number PRJNA1134716 for liquid SE experiments.

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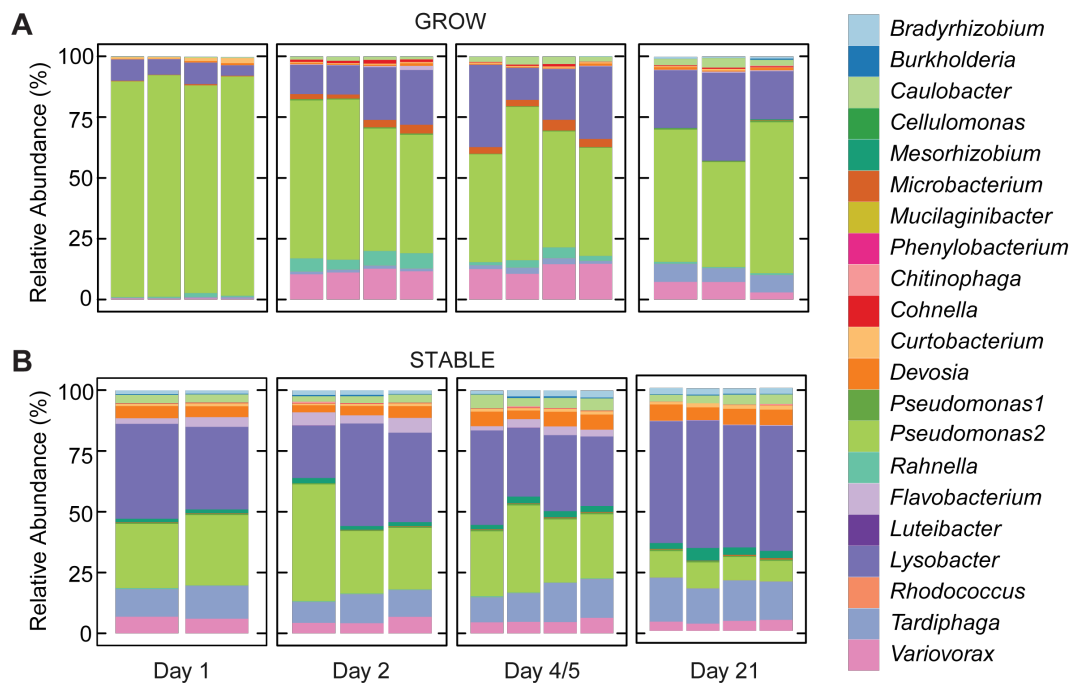
## Supplemental Information

### LIST OF SUPPLEMENTAL FIGURES

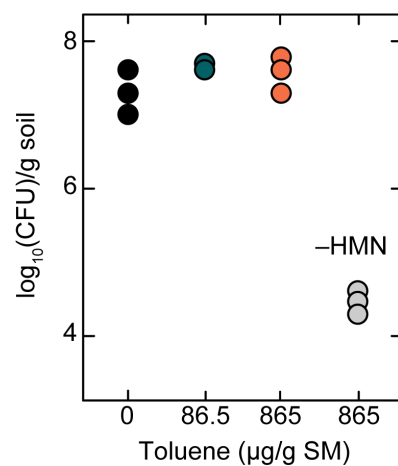
|                                                                                                                                                                                                                                                                                    |     |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| Supplemental Figure 3. 1. Stacked relative abundances of the SynCom strains in soil microcosm.....                                                                                                                                                                                 | 103 |
| Supplemental Figure 3. 2. Cell densities of SynCom 24 h after transfer to fresh soil matrix with varying toluene concentrations.....                                                                                                                                               | 104 |
| Supplemental Figure 3. 3. Stacked relative abundances of the SynCom strains in soil microcosm in A grow and B stable phases with toluene exposure.....                                                                                                                             | 105 |
| Supplemental Figure 3. 4. Non-metric multidimensional scaling analysis of the SynCom species relative abundance data during the grow (dark aqua) and stable (orange) phase of growth over time in the absence (circles) and presence (diamonds) of 130 µg toluene per g soil ..... | 106 |
| Supplemental Figure 3. 5. Stacked absolute abundances of the SynCom strains in soil microcosm with toluene exposure .....                                                                                                                                                          | 107 |
| Supplemental Figure 3. 6. Toluene concentrations over time in soil microcosms without the SynCom in the presence (diamonds) or absence (circles) of <i>P. veronii ptxD<sup>+</sup></i> ..                                                                                          | 108 |

|                                                                                                                                                                                                                                                                                  |     |
|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| Supplemental Figure 3. 7. The effect of phosphite (Phi) on <i>P. veronii ptxD</i> <sup>+</sup> growth and toluene clearance in soil microcosms without the SynCom. ....                                                                                                          | 109 |
| Supplemental Figure 3. 8. Stacked absolute abundances of the SynCom strains and <i>P. veronii ptxD</i> <sup>+</sup> ( <i>Pveronii</i> in the legend) in toluene-contaminated soil microcosm in grow (top) and stable (bottom) phase with and without the application of Phi..... | 110 |
| Supplemental Figure 3. 9. NMDS plot based on Bray-Curtis distances (stress = 0.047) showing the composition of the toluene-remediation system in the presence (diamonds) or absence (circles) of phosphite (Phi). ....                                                           | 111 |
| Supplemental Figure 3. 10. Stacked relative abundance of SynCom members over time in soil extract (SE) medium. ....                                                                                                                                                              | 112 |
| Supplemental Figure 3. 11. Cell densities in soil extract (SE) determined by CFU counting over time. ....                                                                                                                                                                        | 113 |
| Supplemental Figure 3. 12. Stacked relative abundance of SynCom members over time in soil extract (SE) medium in the presence of <i>P. veronii ptxD</i> <sup>+</sup> with and without phosphite (Phi).....                                                                       | 114 |

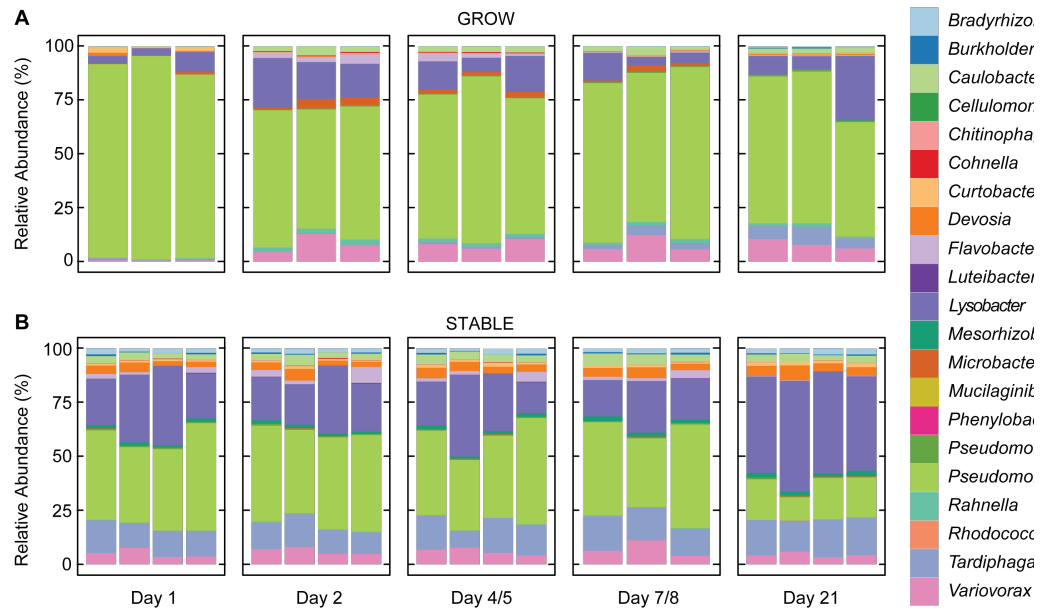




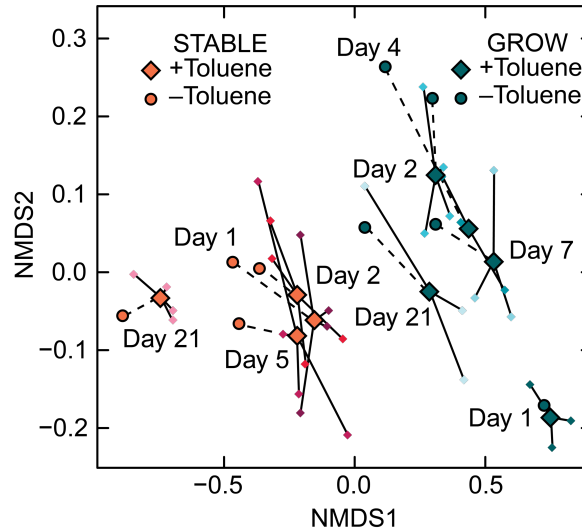
**Supplemental Figure 3. 1.** Stacked relative abundances of the SynCom strains in soil microcosm in **A** grow and **B** stable phase. Grow phase was sampled on days 1, 2, 4, and 21, whereas stable phase was sampled on days 1, 2, 5, and 21. Stacked columns indicate individual biological replicates. SynCom member annotations indicated on the right.



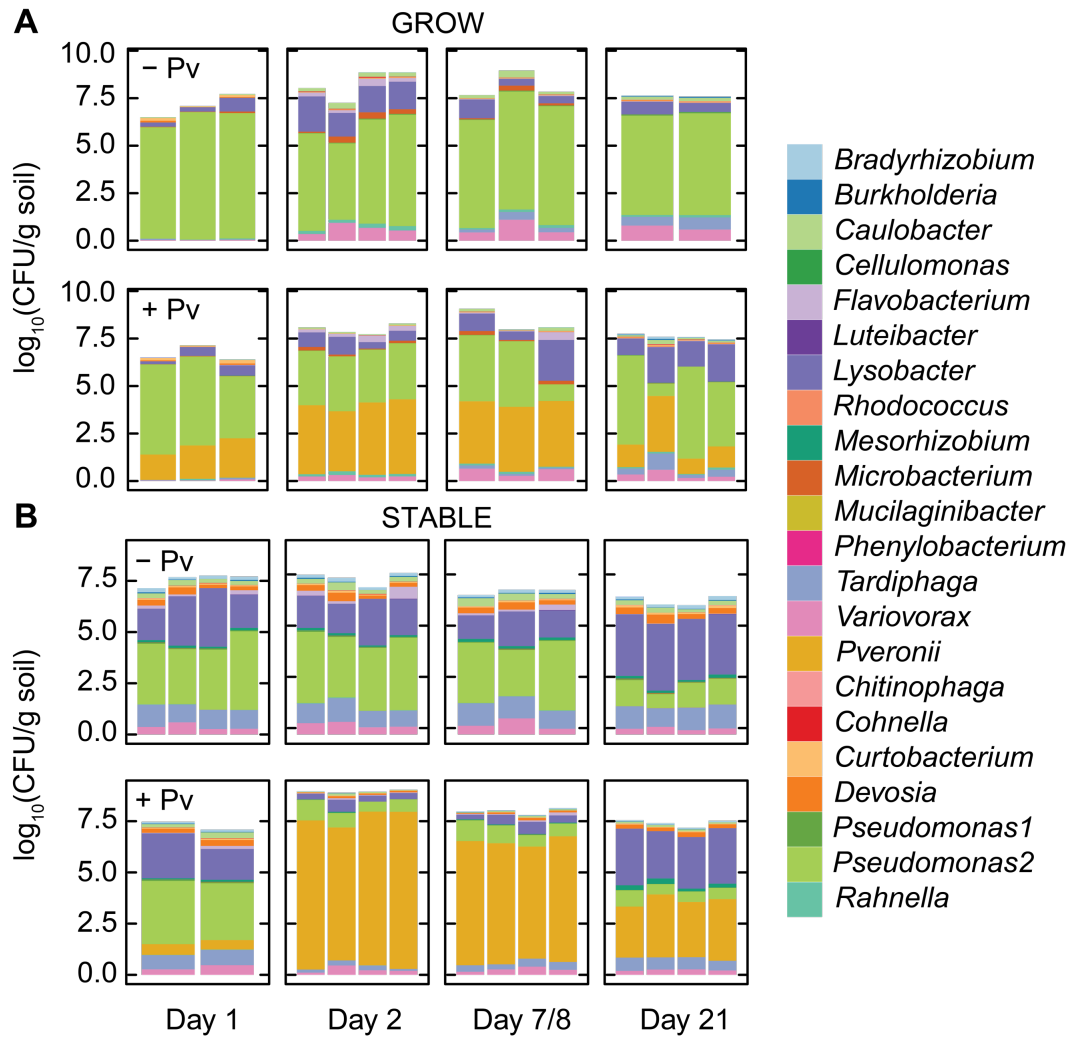
**Supplemental Figure 3. 2.** Cell densities of SynCom 24 h after transfer to fresh soil matrix with varying toluene concentrations: 0 µg/g soil matrix (black); 86.5 µg/g SM (dark aqua); 865 µg/g SM, orange; 865 µg/g SM without 2,2',4,4',6,8,8'-heptamethylnonane (HMN), gray). Toluene was delivered as a solute in HMN at a concentration of 2% v/v in SE, except where indicated (-HMN).



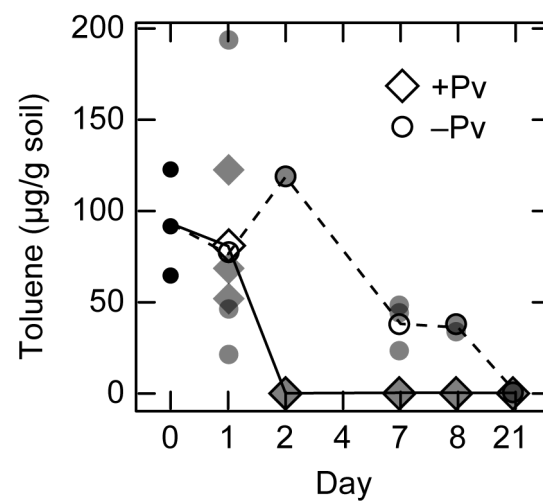
**Supplemental Figure 3. 3.** Stacked relative abundances of the SynCom strains in soil microcosm in **A** grow and **B** stable phases with toluene exposure. Grow phase was sampled on days 1, 2, 4, 7, and 21, whereas stable phase was sampled on days 1, 2, 5, 8, and 21. Columns indicate individual biological replicates. SynCom member annotations indicated on the right.



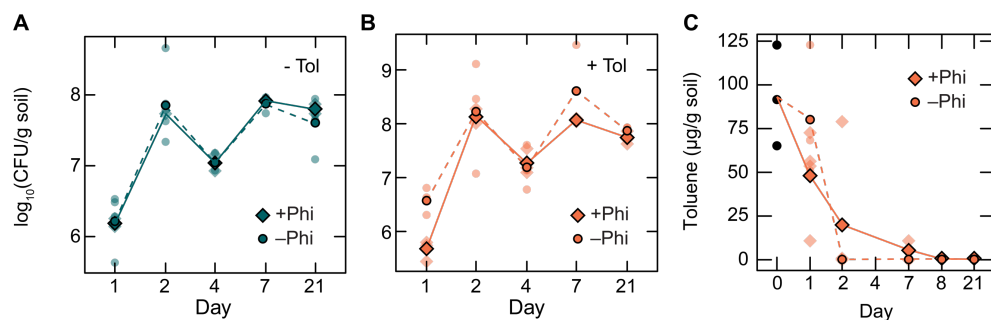
**Supplemental Figure 3. 4.** Non-metric multidimensional scaling analysis of the SynCom species relative abundance data during the grow (dark aqua) and stable (orange) phase of growth over time in the absence (circles) and presence (diamonds) of 130  $\mu\text{g}$  toluene per g soil using Bray-Curtis distance values (stress = 0.045). Data from non-toluene treated samples reproduced from the main text Figure 1. Daily centroids are shown as outlined circles or diamonds and replicates are shown as smaller circles, colored according to timepoint and community phase. Daily centroids are shown as outlined diamonds or circles, and replicates for toluene-treated SynCom are shown as smaller diamonds, colored according to timepoint and community phase.



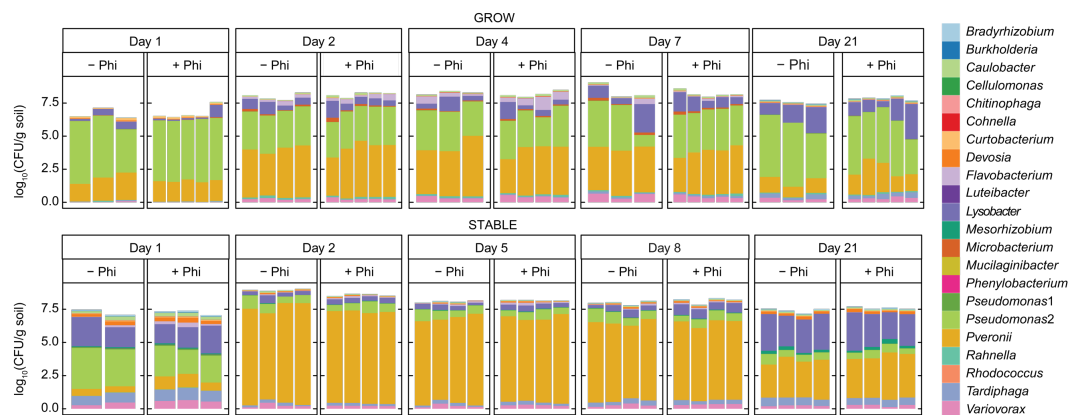
**Supplemental Figure 3. 5.** Stacked absolute abundances of the SynCom strains in soil microcosm with toluene exposure (130 µg/g soil) in **A** grow and **B** stable phases with (+ Pv) and without (– Pv) *P. veronii* <sup>ptxD</sup> bioaugmentation. Grow phase was sampled on days 1, 2, 7, and 21, whereas stable phase was sampled on days 1, 2, 8, and 21. Columns indicate individual biological replicates. SynCom member annotations indicated on the right.



**Supplemental Figure 3. 6.** Toluene concentrations over time in soil microcosms without the SynCom in the presence (diamonds) or absence (circles) of *P. veronii ptxD*<sup>+</sup> ( $p = 0.012$ , two-way ANOVA).

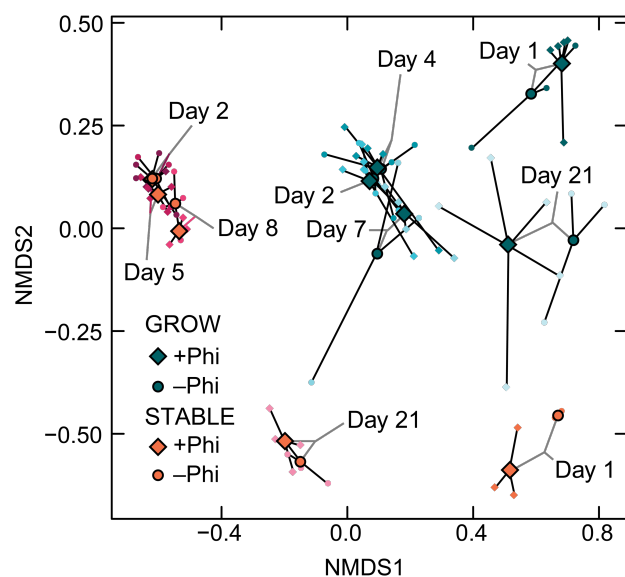


**Supplemental Figure 3. 7.** The effect of phosphite (Phi) on *P. veronii ptxD*<sup>+</sup> growth and toluene clearance in soil microcosms without the SynCom. **A** Absolute *P. veronii ptxD*<sup>+</sup> abundance over time in soil microcosm in the presence (dark aqua diamonds), and absence (dark aqua circles) of 12 µg Phi per g soil. **B** Absolute *P. veronii ptxD*<sup>+</sup> abundance over time in soil microcosm in the presence (orange diamonds), and absence (orange circles) of 12 µg Phi per g soil contaminated with 130 µg toluene per g soil. **C** Toluene clearance over time in the presence and absence of Phi.

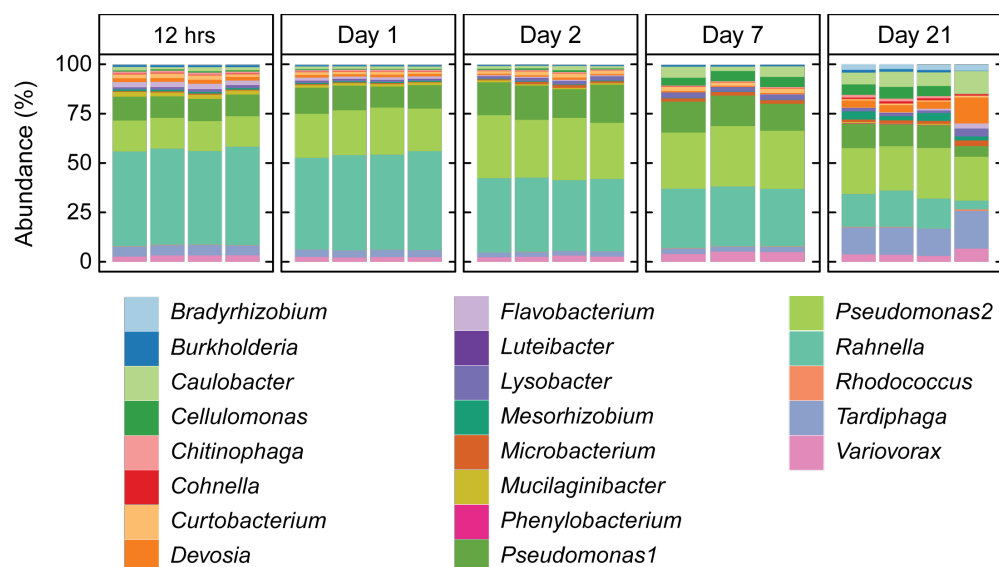


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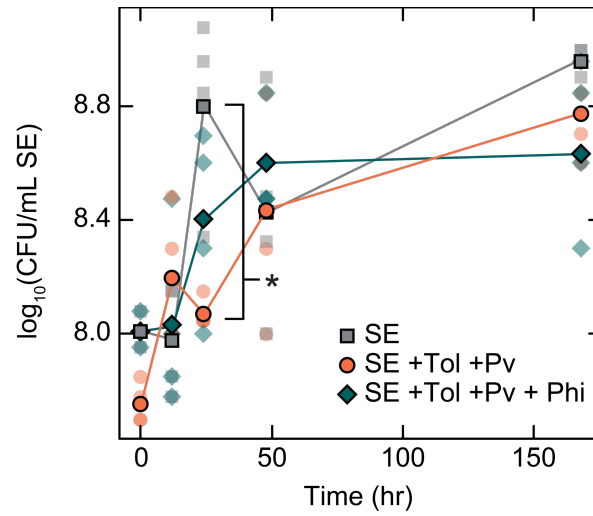




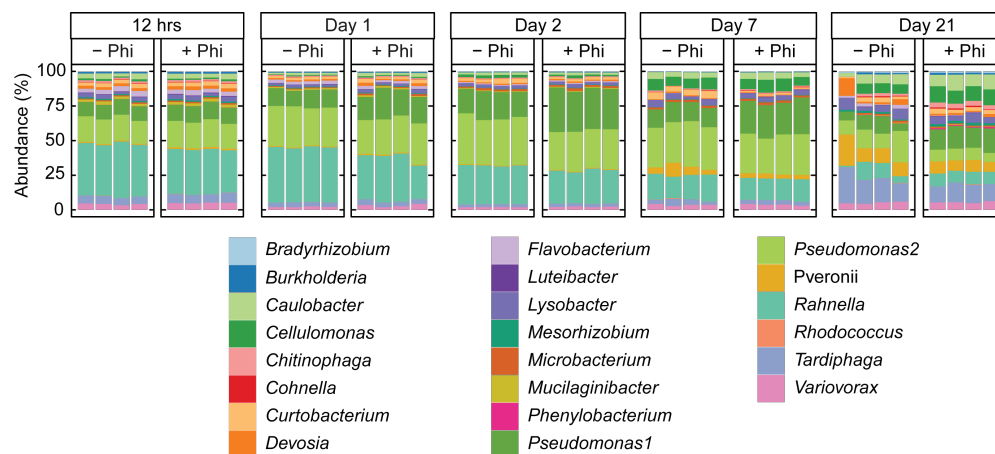
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**Supplemental Figure 3. 10.** Stacked relative abundance of SynCom members over time in soil extract (SE) medium. Columns indicate individual biological replicates. SynCom member annotations indicated on the bottom.



**Supplemental Figure 3. 11.** Cell densities in soil extract (SE) determined by CFU counting over time. The SynCom community growth alone is represented with gray squares. The SynCom growth in the presence of 600  $\mu$ g toluene per mL with *P. veronii ptxD*<sup>+</sup> in toluene-contaminated SE with (1.3 mM, orange circles) and without Phi (dark aqua diamonds) are also shown. Significant difference in cell densities between SynCom in SE and SynCom and *P. veronii ptxD*<sup>+</sup> in toluene-contaminated SE at 24 h is shown ( $p = 0.018$ , Kruskal-Wallis test).



**Supplemental Figure 3. 12.** Stacked relative abundance of SynCom members over time in soil extract (SE) medium in the presence of *P. veronii* *ptxD*<sup>+</sup> with and without phosphite (Phi). Columns indicate individual biological replicates. SynCom member annotations indicated on the bottom.

## V. Conclusions

The P biogeochemical cycle features a significant, but understudied P pool in a reduced ( $<+5$ ) form that is suspected to be of biological origin. Phi, hypothesized to be linked to the biosynthesis of phosphonates, could provide a link between biological Pi assimilation and the ultimate release of phosphine gas in aerobic environments, “closing” the P cycle. In this work, I have advanced our ability to interrogate this question through the methodological development of a Phi sensor that shows robust performance for an array of relevant biological and environmental matrices. I then applied this fluorometric sensor, along with  $^{31}\text{P}$ -NMR, to detail a Phi-producing mechanism in *S. viridochromogenes*. This mechanism, involving the activated phosphonate intermediate, PF-CMP, may be only one of many biological Phi-producing pathways, however, it provides insight on the chemical logic involved in this chemistry that can aid in future bioinformatics-based studies in order to detail the breadth and scale of Phi production through nucleotidyl activation. Finally, Phi was assessed as a higher solubility and selective P source for a toluene-degrading inoculant, *P. veronii*. This provided insight into the efficacy of a Phi-directed remediation system, which is highly dependent on low bioavailable P in the surrounding environment. Through these investigations, I hope to draw attention the importance of Phi in the biogeochemical Phi cycle and open new lines of investigation into role of microbially-produced Phi in laboratory culture and the environment.