Redox-Active Antibiotics Enhance Phosphorus Bioavailability

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Abstract

Microbial production of antibiotics is common but our understanding of their roles in the environment is limited. Here, we explore long-standing observations that microbes increase production of redox-active antibiotics under phosphorus limitation. The availability of phosphorus, a nutrient required by all life on Earth and essential for agriculture, can be controlled by adsorption to and release from iron minerals via redox cycling. Using phenazine antibiotic production by pseudomonads as a case study, we show that phenazines are regulated by phosphorus and solubilize phosphate via reductive dissolution of iron oxides in the lab and field, concomitantly increasing microbial growth. Phenazines are just one of many examples of phosphorus-regulated antibiotics. Our work suggests a widespread but previously unappreciated role for redox-active antibiotics in phosphorus acquisition and cycling.

One Sentence Summary:
Antibiotics enhance P bioavailability.

The production of secondary metabolites by microbes is widespread, and there is growing recognition of the importance of these molecules for survival in the wild (1–3). However, with the exception of metal complexation by siderophores, the controlled use of secondary metabolites in nutrient acquisition has not been shown. In response to limitation for the essential nutrient phosphorus (P), several bacterial species increase production of secondary metabolites (4, 5). Many of these metabolites are considered to be antibiotics, and their toxicity is conferred by a variety of mechanisms, including the ability to engage in redox reactions. P-regulation of secondary metabolite production has been studied extensively in Actinobacteria but also occurs in Proteobacteria and Firmicutes (Fig. 1). The reason for this regulation is not totally understood but, changes in cell metabolism under slow growth and nutrient stress as well as a broad phosphate-starvation induced virulence response have been

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Supplementary Materials: Materials and Methods
suggested (5, 6). An alternate, unexplored explanation is that under P limitation, rather than having toxic roles, the redox activity of some of these antibiotics might directly facilitate P acquisition.

Phosphorus availability affects global primary productivity in natural and agricultural systems (7–10) and can be influenced by microbial redox reactions involving iron (Fe) oxides over geologic as well as anthropologic time scales (7, 8, 11, 12). Phosphate and organic P are often immobilized via adsorption to positively charged surface sites on Fe(III)-(oxy)hydroxide minerals and subsequently solubilized by microbial reduction of Fe(III) to Fe(II) under anoxic conditions (7, 11–13). The controls on microbial P solubilization from Fe oxides and their contribution to P burial remain an under-constrained facet of the P cycle. Fe reduction by metabolites such as flavins (which are not known to be regulated by P) has been well studied (14). However, the primary purpose of these metabolites is thought to be respiration of Fe minerals, and any accompanying benefit from P solubilization has either been neglected or considered perfunctory.

Based on the precedent for P release via reductive dissolution of Fe oxides, we wondered if some antibiotics with redox activity might have a previously unappreciated role in this process, thus reconciling their stimulation by P limitation. We set out to quantify the stimulation of redox-active antibiotic production under P limitation and examine the resulting enhancement in P availability and microbial growth. Specifically, we sought to test the following hypotheses: i) redox-active antibiotic biosynthesis is regulated by P availability in many organisms and occurs via a conserved molecular pathway, ii) reductive dissolution of Fe minerals by these metabolites results in solubilization of adsorbed P and iii) the net result is increased P availability in lab cultures and natural microbial communities under P limitation.

We focused on phenazines (Fig. 1), a class of secondary metabolites with well understood redox properties (15) that are made by several pseudomonads as well as many other types of bacteria and are known to confer a variety of physiological benefits to the producing organism (3, 16). Phosphorus limitation was linked to phenazine production in *Pseudomonas aeruginosa* as early as 1947 (17), but it has been unclear whether this is widespread in pseudomonads. Additionally, when studied in batch culture, secondary metabolites are typically produced at the end of growth – due to a complex combination of nutrient exhaustion as well as the accumulation of quorum sensing (QS) molecules, which regulate biosynthesis of many secondary metabolites, including phenazines (18).

Chemostats provide a more controlled experimental setup than batch culture, allowing for precise tuning of both growth rate and cell density, thereby removing these complications. We designed a growth medium that maintains low cell densities (OD500 circa 0.1) based on limitation for either nitrogen or phosphorus and quantified phenazine production under these conditions in *P. aeruginosa, P. aureofaciens, P. chlororaphis* and *P. fluorescens*. We found that in all four *Pseudomonas* species tested, chemostats limited for phosphorus had much higher phenazine concentrations than those limited for nitrogen, ranging between 1 and 3 orders of magnitude. Slow growth rates also correlated with greater phenazine production (Fig. 2A, Fig S1), an observation that has been made by others (4, 19) but is not completely
These results are consistent with previous chemostat experiments in *P. aeruginosa* (19) as well as batch culture findings (4, 17, 18, 20) and show widespread enhancement of phenazine production under P limitation in pseudomonads.

In many bacteria, genes related to phosphorus acquisition are part of the PHO regulon, which is controlled by a two-component regulatory system comprising an inner membrane histidine kinase (PhoR) and a cytoplasmic response regulator (PhoB) that controls transcription by binding to conserved regulatory ‘PHO boxes’ (Fig. 2C, (6)). To test whether PhoB regulation might explain the trends seen in our *Pseudomonas* chemostat experiments, we constructed unmarked deletions of the phoB gene in *P. fluorescens* and *P. chlororaphis*. In both cases, the deletion of phoB abolished phenazine production in phosphorus-limited chemostats (Fig. 2D). These results are supported by previous findings that a *P. aeruginosa* phoB mutant produces less of the phenazine pyocyanin under P limitation and as well as in silico predictions of putative PHO boxes upstream of phenazine biosynthetic genes and QS genes (21). When considered with prior studies (Fig. 1, Table S1, (22)), our findings suggest that PhoB regulation of secondary metabolite production is conserved not only in pseudomonads but also across diverse species.

Phenazines are known to reduce Fe minerals (15) but, the potential effects of this process on P solubilization have not been investigated. To test whether phenazines could solubilize phosphate via reductive dissolution, phenazines were added to synthetic phosphated hydrous ferric oxides (herein HFO-P), and the release of phosphate (as elemental P), total Fe, and Fe(II) was tracked. Reduced 1-hydroxy-phenazine (1-OH-PHZ), phenazine-1-carboxamide (PCN) and phenazine-1-carboxylic acid (PCA) increased soluble phosphorus concentrations relative to controls, whereas oxidized forms of these phenazines did not (Fig. 3A). In addition, Fe(II) was only produced in incubations with reduced phenazines (Fig. 3A), consistent with HFO-P reductive dissolution, a mechanism that has been well established as an important control of environmental P availability (12).

To examine whether phenazines would stimulate microbial growth of P-limited cultures on HFO-P, we focused on 1-OH-PHZ and PCA as these phenazines are most reactive with Fe oxides and are made by soil and sediment dwelling pseudomonads (15, 23, 24). A *P. aeruginosa* mutant that cannot make phenazines was cultured under Fe-replete conditions with HFO-P as the sole P source. While this organism is best known for its role as an opportunistic pathogen, phenazine-producing *P. aeruginosa* strains have also been isolated from coastal marine sediments (24). To avoid oxidation of phenazines by oxygen and to provide an alternative electron acceptor, cells were grown anaerobically with nitrate. The addition of either reduced PCA or reduced 1-OH-PHZ increased growth (Fig. 3C). A further increase in growth was observed upon addition of P in the presence of phenazines (Fig. 3C), validating P limitation under these conditions. These results establish that reduced phenazines stimulate P-limited cultures in the presence of HFO-P, demonstrating their ability to increase the bioavailability of P (and possibly Fe) from the particulate phase.

To extend these findings to the environment, we conducted anaerobic incubations of phenazines with soils (Site 1: 33° 26.1008’ N, 118° 30.1868’ W, August 2019) and sediments (Site 2: 33° 25.7952’ N, 118° 30.2765’ W, August and October 2019) collected.
from Catalina Harbor on Catalina Island, CA, a location where pseudomonads have been previously isolated (25). Our experiments showed a variety of P solubilization responses to phenazine additions (Fig. 4), ranging from no change to significant increases; and these differences were correlated with variations in Fe:P ratios of soil/sediments. Experiments from Site 1, which had a very high Fe:P ratio of ~88 (Fig. 4A), showed little response to phenazine additions (Fig. 4B). Oxidation of a large fraction of added PCA by Fe at P-free mineral surface sites and re-adsorption of solubilized P could explain these muted effects. In contrast, at Site 2, where the Fe:P ratio was substantially lower (~26, Fig. 4A) reduced PCA significantly increased total soluble phosphorus in two separate experiments, but with very different temporal dynamics (Figs. 4C,D, S3, S4). In the first set of experiments conducted in August 2019, reduced PCA led to an immediate and sustained elevation in phosphorus concentration. In experiments from October 2019, reduced PCA led to dramatic but delayed increases in soluble phosphorus starting at 72 hours. Experiments with 1-OH-PHZ at Site 2 in August showed little effect (Fig. S3, S4), likely owing to greater reactivity of 1-OH-PHZ (15) and subsequent formation of secondary mineral phases.

Measurements at the end of experiments (see Methods) showed the presence of sulfide in incubations from October but not August. Increased activity of sulfate reducing bacteria (SRB) explains the sharp increase in P solubilization seen later in the year as sulfide promotes reductive dissolution of Fe(III) oxides and release of sorbed P (26). Fe(III) reduction in the October incubations was directly observed as a peak in dissolved Fe(II) at 72 hours (Fig. S5), followed by a decrease as expected from the subsequent precipitation of Fe(II)-S phases with increasing S(-II) concentration. Interestingly, sulfide was found only in reduced PCA treatments, suggesting reduced PCA indirectly increases P solubilization by stimulating sulfate reduction, possibly by assuaging SRB limitation for Fe and/or P (27). Overall, our Catalina Island experiments show that phenazines can solubilize P from natural sediments through both direct and indirect processes.

A potentially advantageous feature of redox-active metabolites is their capacity to be re-reduced and cycled by the microbial community (16). There is some evidence for this occurrence in our experiments at Catalina Island: when oxidized phenazines were added (Figs. S3, S4), treatments showed modest increases in soluble phosphorus over time, consistent with P solubilization via phenazine that was reduced in situ. To test this possibility, we tracked the reduction of PCA in samples of sediments (using a fluorescence assay, see Methods) from Site 2 and either suppressed biological activity with ethanol or stimulated it with organic carbon. Ethanol dramatically suppressed PCA reduction (Fig. 4E) while organic carbon stimulated it (Fig. 4F), as expected for a biologically-driven process. The capacity of native microbial communities to reduce phenazines is notable as it suggests a small concentration of such metabolites could have a large effect on P solubilization.

In conclusion, we demonstrate an ecophysiological role for redox-active antibiotics in phosphorus solubilization and acquisition. This phenomenon is potentially widespread since, like phenazines, phosphate-regulated metabolites including tetracyclines (which can reduce ferrihydrite (28)) and those with putatively redox-active moieties (quinone, phenoxazine, Fig. 1, Table S1) may also solubilize P via reductive dissolution. Given that global reserves of mineable phosphorus rock, the source of P fertilizers, are predicted to be exhausted
shortly (9), and that phenazine producers are found in crop soils (23) our findings in marine sediments may also have applied relevance for the P cycle in agricultural contexts. Overall, this work expands our knowledge of beneficial physiological roles for antibiotics, indicating that they also contribute to the acquisition of macronutrients and biogeochemical cycling.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. P regulates production of antibiotics in diverse bacteria.

Tree depicts species with experimental evidence for P-limited antibiotic production (pink text) and experimentally confirmed (filled green circle) or likely (open green circle) regulation by phoB/P and phoR. Data are largely from (5) and (6), see Table S1. Metabolite production in B. thailandensis and Serratia ATCC39006 was tested using chemostats (Fig. S1). Example structures for different antibiotics are shown with broad antibiotic type listed above each structure. The common metabolite name and producer are also listed. Producers of each example metabolite are also indicated with numbers around the tree. Tree was built from 241 small subunit rRNA sequences using RaxML (29) and is rooted for display.
Fig. 2. Phenazine production in different pseudomonads is regulated by phosphate via \textit{phoB}.

(A) Phenazine production in phosphorus- (blue) or nitrogen- (yellow) limited \textit{Pseudomonas} chemostats. Phenazines produced are PCA: phenazine-1-carboxylic-acid; PCN: phenazine-1-carboxamide; PYO: pyocyanin. ODs were maintained at ~0.1, the range of ODs across chemostats for each experiment ± standard deviation is listed. (B) Phenazine structures. (C) PhoB is thought to increase phenazine production by binding predicted PHO boxes upstream of phenazine biosynthetic genes and QS genes (19, 21). (D) Phenazine production in wild type \textit{Pseudomonas} and \textit{phoB} mutants at $\mu=0.06$. Reported growth rate ($\mu$) is the dilution rate, the two are equivalent at steady state. Nd: not detected, OD: optical density at 500 nm.
Fig. 3. Phenazines solubilize P and promote *P. aeruginosa* growth on HFO-P.

(A) Results of reactions between HFO-P and phenazines after 5 hours of anoxic incubation: Fe(II) (ferrozine assay), total soluble iron (ICP-MS), and total soluble phosphorus (ICP-MS). Nd: not detected. Error bars: standard deviations for duplicates. See Fig. S2 for details on P adsorption. (B) Model of reductive dissolution. The reaction of phenazine with Fe(III) is a two electron transfer yielding 2Fe(II) (15). Solubilized P is variable (indicate by n) depending on the extent of P surface coordination as dictated by P concentration, mineral composition, and pH (26). (C) Growth (as a denitrifier) of a *P. aeruginosa* mutant unable to make phenazines on HFO-P. Additions: 100 μM (reduced phenazine), 7mM (phosphate). Shaded area represents the standard deviation for biological duplicates.
Fig. 4. Phenazines solubilize P in marine sediments.

(A) Nitric acid extractable iron and phosphorus from sampling sites. Error bars: standard deviations from triplicate digestions. (B-D) Phosphorus solubilization in Catalina Island sediments from Site 1 collected in August (B), and Site 2 collected in August (C) or October (D). Y-axes in (B-D) reflect difference in total soluble phosphorus (ICP-MS) from the initial time point, horizontal lines depict no change. See also Figs. S3, S4. (E) PCA reduction is suppressed in sediments treated with ethanol. (F) PCA reduction is stimulated by organic carbon (10 mM glucose + 10 mM lactate). Sediments in (F) were starved before PCA additions and cannot be compared directly to (E). For (B-F) Plots reflect data from 4 or 5 replicates, outliers (single black dots) are >1.5x interquartile range, p values reflect...
comparison to the control treatment (as indicated on the figure) at a specific time point. *
$p<0.05$, ** $p<0.005$, *** $p<0.0001$. 