Microbial Phosphorus Acquisition: A Tale of Drugs, Flowers, and Metal

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I. Background

Phosphorus, along with carbon, nitrogen, and sulfur, is an essential nutrient for microbial growth. However, in many environments, phosphorus is a limited resource, either due to biological consumption or chemical unavailability. Accordingly, microbes have evolved different strategies to efficiently acquire phosphorus from the environment. This mini-report uses three, separate approaches to investigate mechanisms of how microbes overcome phosphorus limitation:

A. Drugs

Phosphorus is found most abundantly in nature as phosphate, and many microbes readily consume phosphate as a sole source of phosphorus. Competition for phosphate is likely to be highly intense in most environments. The ability to consume sources of phosphorus other than phosphate [1,2], especially compounds that are recalcitrant and unavailable to competitors, may therefore provide a significant benefit in phosphate-limited environments. Antibiotics, such as fosfomycin produced by *Streptomyces* species [3], may represent one such class of compounds. Catabolism of fosfomycin and other antibiotics may also serve as a powerful mechanism of antibiotic resistance. The goal of this mini-project was to enrich for and isolate microbes that can use fosfomycin as a sole source of phosphorus. Parallel enrichments targeted microbes that can use antibiotics as a sole source of carbon, nitrogen, or sulfur.

B. Flowers

The rhizosphere of plants are densely colonized with microbes that directly form biofilms on the surfaces of plant roots [4]. In many cases, this association is mutually beneficial – for instance, when the plant provides nutrients for the microbe, while the microbe protects the plant from invading pathogens [5]. One such biocontrol agent is *Pseudomonas fluorescens*. Biofilm formation by *P. fluorescens* is strongly regulated by phosphate levels. In general, high phosphate conditions promote biofilm formation, while low phosphate conditions prevent biofilm formation [6]. However, phosphate is not only an important source of phosphorus for *P. fluorescens* but also for the plant host, raising the issue of whether *P. fluorescens* directly competes with plant hosts for phosphate [7]. Moreover, if *P. fluorescens* does compete with plant hosts for phosphorus, why does this interaction not abolish biofilm formation by *P. fluorescens* on plant roots? To address these questions, one approach is to use *P. fluorescens* as a bio-reporter of phosphate availability in the rhizosphere. If phosphorus is limiting, then low phosphate-responsive genes should be activated, while high-phosphate responsive genes should be suppressed. The reverse should occur if phosphorus is abundant. An approach to address this question with high spatial resolution is hybridization chain reaction (HCR)-FISH, a novel technique for visualizing the expression of specific transcripts in single cells [8]. With HCR-FISH, questions such as where along the plant root and at what distance away from the plant root does the bacterium experience phosphate starvation, if at all, can be asked. The goal of this mini-project was to colonize the flowering plant *Arabidopsis thaliana*, a model for plant genetics and plant-microbe interactions [9], with *P. fluorescens* and to use HCR-FISH to examine the spatial distribution of phosphate-regulated gene expression in the *Arabidopsis* rhizosphere.

C. Metal

Phosphate is often chemically unavailable because it adsorbs to the surface of iron hydroxide particles. Reduction of these particles, however, can release adsorbed phosphate back into the environment. Microbes may have therefore developed strategies to carry out this process biologically [10]. The goal of this mini-project was to investigate the potential for phosphate-regulated manganese reduction by a novel Actinomycete.

II. Materials and Methods

Enrichment for antibiotic users. A modular medium to enrich for antibiotic users contained:

Component	Concentration
Freshwater base*	1x
Trace elements*	1x
Sodium succinate (C source)	10 mM
Ammonium chloride (N source)	10 mM
Sodium sulfate (S source)	1 mM
Potassium phosphate (P source)	1 mM
MES buffer (pH 5.5)	10 mM
Cycloheximide	50 μg mL^{-1}
*See Appendix for components.	

Where appropriate, the C, N, S, or P source was replaced with an antibiotic. Antibiotics were made up as 10x stock solutions in ddH_2O and added to the media after autoclaving. Soil inocula were collected from in front of the Loeb, Ebert, and Swope buildings in Woods Hole, MA and mixed together. Approximately 100-200 mg of soil was added to 5 mL modular medium in a test tube for each enrichment. Cultures were incubated at room temperature. As a control, cultures were also prepared without a C, N, S, or P source added. Cultures were transferred by diluting 1:100 into fresh media. Plates for single colony isolation were prepared with 1.5% agar in the medium.

Hybridization chain reaction. P. fluorescens Pf0-1 strains for HCR-FISH were provided by George O'Toole. Strains were routinely cultured in LB or K10 minimal medium (see Appendix for composition) at 30˚C with vigorous shaking. The *pst*⁻ strain was maintained with 30 μg mL^{-1} gentamicin in the medium. Prior to fixation, strains were diluted 1:100 or 1:1,000 (depending on growth rate) and cultured until mid- to late-exponential phase (roughly, Abs_{600} 0.4-0.6). Growth under low phosphate was in K10 with no phosphate added to the medium, and growth under high phosphate was in K10 with 10 mM phosphate added to the medium. Fixation, hybridization, and amplification procedures (below) were carried out essentially as described in the 2014 Microbial Diversity lab manual.

Fixation: Two fixation procedures were tested for FISH. Cells fixed with 4% paraformaldehyde (PFA) were fixed for 1 hour at 4˚C, centrifuged for 3 min at 4300 x g at room temperature (RT), resuspended in 1 mL 0.02x SSC (see Appendix), centrifuged for 3 min at 2000 x g, resuspended in 1 mL 0.5 M glycine, incubated for 5 min at RT, centrifuged for 3 min at 2000 x g, resuspended in 100 μL 1x PBS, and mixed 1:1 with ice cold ethanol. Cells fixed with 1% PFA were fixed for 30 min at RT, centrifuged for 5 min at 3700 x g at RT, resuspended in 1x PBS, centrifuged again, resuspended in 200 uL methanol, and permeabilized for 1 hour on a vortexer. Fixed cells were stored at -20˚C.

Slide preparation: Fixed cells (10-20 µL) were applied to a poly-l-lysine coated slide and allowed to dry. Cells were dehydrated by successively flooding slides with 50%, 80%, and 98% ethanol for 3 min each.

Probes:

Gene target	Probes	Hairpins	Fluorophore	Inducing signal
Alkaline phosphatase	B1D1 1-5	B1H1, B1H2	Alexa488	Low phosphate
GlpT	B2D1 1-5	B2H1, B2H2	Alexa546	High phosphate
LapG	B2D1 1-5	B2H1, B2H2	Alexa546	Constitutive
LapD	B1D1 1-5	B1H1, B1H1	Alexa488	Constitutive

Probe hybridization: Probe hybridization buffer (PHB, see Appendix) was pre-heated to 45˚C, and the probes were added to a final concentration of 2 nM (1 µL of each 1 µM probe per 500 µL) and inverted repeatedly to mix. Slides were pre-hybridized with 50 µL PHB at RT for 10 min. PHB was removed by tilting the slides onto Kim-Wipes and blotting. Slides were applied with 20 µL probe solution and coverslipped. Slides were incubated overnight at 45˚C in humidified chambers (either in 50 mL Falcon tubes or empty pipette boxes with moist paper towels lining the bottom).

Probe washes: Slides were washed in Coplin basins. Slides were successively washed for 15 min each at 45˚C with 75% PWB / 25% 5x SSCT, 50% PWB / 50% 5x SSCT, 25% PWB / 75% 5x SSCT, 100% 5x SSC. Wash buffers were re-used and stored at 4˚C between experiments.

Hairpin amplification: Hairpins were snap cooled by aliquotting each hairpin into separate PCR tubes, heating to 95˚C for 90 seconds, and cooling in the dark at RT for at least 30 min. After the washes, slides were blotted with Kim-Wipes and pre-amplified with 50 µL amplification buffer (see Appendix) for 5 min at RT. The hairpins were added to amplification buffer at a final concentration of 60 nM (10 µL of each 3 µM hairpin per 500 µL) and inverted repeatedly to mix. The slides were blotted, applied with 20 µL hairpin solution, and coverslipped. Slides were incubated overnight at RT in a humidified chamber.

Hairpin washes: Slides were washed in Coplin basins. Slides successively washed in 5x SSC for 15 min each at RT.

Microscopy: Slides were mounted with a DAPI:Vectashield®:Citifluor solution and examined with a Zeiss microscope (model: really nice)

Arabidopsis. A. thaliana seeds of ecotype Col and MS (Murashige & Skoog) mineral salts were provided by Dr. Joseph Chen of The University of Texas at Austin. MS media contained 4.3 g MS salts, 30 g sucrose, and 8 g agar per 1 L ddH_2O (pH 5.7). Seeds were sterilized with ethanol and bleach. A small scoop of seeds was transferred to a 1.5 mL microcentrifuge and soaked in 1 mL sterile ddH_2O for 30 minutes, 1 mL 80% ethanol for 1 min, and 1 mL bleach for 10 min. Seeds were rinsed 7 times with 1 mL sterile ddH_2O. Seeds were plated onto MS agar, parafilmed, and incubated at 4˚C for 3 days to synchronize germination. Seeds were transferred to 50 mL Falcon tubes with 20 mL fresh MS medium and incubated at RT on a windowsill under a steady source of lamplight. Media for colonizing germinated plants with *P. flourescens* was prepared by lowering the agar to 0.3% and allowing the media to cool after autoclaving. An overnight of *P. fluorescens* (grown in liquid MS media) was inoculated into the media at a concentration of 10^7-10^8 CFU mL^{-1}. Once the media hardended, germinated *Arabidopsis* plants were transferred from liquid MS onto solid MS inoculated with *P. fluorescens*. *P. fluorescens* was allowed to colonize the plants for 1 day before fixing plants for FISH. Colonized plants were

washed with 1x PBS and fixed with 4% PFA overnight at 4˚C. The HCR-FISH procedure was carried out in 1.5 mL microcentrifuge tubes. Plants were transferred between washes with forceps (in general, by gripping the leaves). Plants were mounted in DAPI:Vectashield®:Citifluor and coverslipped.

Actinomycete isolation. Actinomycetes were isolated from environmental samples using standard methods by Cristian Salgado (strain CS-1) and Richard Mariita (strain RM-1).

Manganese reduction assay. Plates for testing for manganese reduction were made with starch arginine tryptophan [11] media as a base. SAT contained:

Component	Concentration
Freshwater base*	1x
Trace elements*	1x
Starch	10 g L^{-1}
Arginine	1.0 g L^{-1}
Tryptophan	0.1 g L^{-1}
Sodium sulfate	0.2 mM
Potassium phosphate	0.1 mM
MOPS buffer (pH 7.2)	1 mM
Multivitamin mix	10 mM
*See Appendix for components. The vitamin mix was added after autoclaving.	

Manganese plates were made by overlaying solidified SAT agar with SAT agar containing 50 mM manganese oxide. The upper layer was prepared with increasing concentrations of potassium phosphate, from 0 to 10 mM. Bacterial strains were cultured overnight in SAT liquid medium at 30℃ with shaking. In the morning, strains were sub-cultured in SAT with 0.1 mM potassium phosphate added (SAT+P) and in SAT without potassium phosphate added (SAT-P) to starve cells of phosphate. *P. fluorescens* was diluted 1:1,000 into the SAT media, while the actinomycete strains, due to slower growth, were pelleted, washed in SAT-P, and resuspended in the SAT media to maximize final biomass for the assay. Strains were sub-cultured for 9 hours prior to spotting the liquid cultures onto manganese plates. Plates were incubated at 30℃ for greater than 1 week prior to capturing images. Little difference was observed between cells starved and not starved of phosphate prior to spotting.

Microelectrode. The pH of different locations on manganese plates after bacterial growth was measured using a pH electrode (thanks, Verena). A location where bacterial growth had not occurred was used for the reference electrode.

Ferrihydrite. Poorly crystalline iron oxides (PCIO) were prepared as previously described [12] with several modifications. 27.05 g of $FeCl_3 \cdot 6H_2O$ were dissolved in 250 mL DI H_2O, brought up to just below pH 7 using 1 M NaOH dropwise while stirring, and left to stir for 30 min. The slurry was washed three times by centrifuging at 5000 rpm for 15 min and resuspending in DI H_2O. Iron oxides were stored at RT in fresh DI H_2O. Phosphate adsorption was carried out as previously described for arsenic [13]. PCIO was mixed 1:1 with potassium phosphate (pH 7.2), stirred overnight, and washed three times by centrifuging at 5000 rpm for 15 min and resuspending in DI H_2O.

Ferrihydrite reduction assay. Actinomycete CS-1 and *P. fluorescens* were grown in liquid SAT under low phosphate (0 mM) and high phosphate (10 mM) conditions. In addition, *P. fluorescens* was grown in an anaerobic chamber under the low and high phosphate conditions with or without nitrate (400 µM) added to stimulate respiratory growth. The aerobic cultures were transferred to the anaerobic chamber and incubated for 2 hours to promote re-reduction of any oxidized secreted compounds in the culture media. The reduced cultures were filtered through

0.45 µm filters, and the supernatants were mixed 1:1 with ferrihydrite and incubated for 1 hour to promote ferric iron reduction. Ferrous iron was quantified using the ferrozine assay.

Molybdenum blue assay. A colorimetric assay for quantifying phosphate levels was modified from Johnson & Pilson, 1972.

Ferrozine assay. A colorimetric assay fro quantifying ferrous iron levels was modified from Stookey, 1970 [14]

III. Results and Discussion

A. Drugs

Previous work has shown that microbes can use antibiotics as a sole source of carbon (C) [15]. Since many antibiotics also contain nitrogen (N), sulfur (S), and/or phosphorus (P), we hypothesized that antibiotics can also serve as a sole N, S, or P source. To test this hypothesis, we designed a modular medium (see Materials & Methods) to enrich for antibiotic users. In designing this medium, we decided to exclude vitamins to reduce trace amounts of C, N, S, or P other than the provided antibiotic. Cycloheximide was include to suppress eukaryotic fungal growth, and the pH was buffered to 5.5 to mimic the pH environment of soil. To narrow the spectrum of tested antibiotics, we focused on classes not previously tested [15], with the exception of penicillin. Penicillin was intended to serve as somewhat of a positive control since independent reports have been made of bacteria using penicillin as a sole carbon source [15,16]. Additional controls for the enrichment included a culture with succinate, ammonium, sulfate, and phosphate as the sole C, N, S, and P sources as well as cultures without an added C, N, S, or P source.

Several days after inoculation, growth (turbidity) was observed for the S and P enrichments, but less so for the C and N enrichments. Although some growth did appear in cultures lacking a C, N, S, or P source, importantly, growth in the enrichments seemed greater. Multiple antibiotic concentrations were tested for the S and P enrichments, and interestingly, growth seemed to scale for the S enrichments with the amount of antibiotic included in the medium, most notably for polymyxin B (Fig. 1). In contrast, little growth was ever observed in the C or N enrichments throughout the entire enrichment procedure. This was surprising considering the previous reports of microbial antibiotic consumption. Microscopic examination, however, revealed several motile forms and diverse morphologies in all cultures (data not shown), including the C and N enrichments. Larger protists were also observed, raising the possibility that grazing prevented abundant bacterial growth in the C and N enrichments. The S and P enrichments were transferred once before plating the P enrichment onto solid media with fosfomycin as the sole P source and, as a control, media without an added P source. Unfortunately, growth was not exclusive to the fosfomycin plate (Fig. 1), indicating that growth with fosfomycin as the sole P source was unlikely. As a future direction, enrichments could be further transferred before plating onto solid media to ensure minimal carryover of C, N, S, or P from the soil inocula. Moreover, the agar in the solid media could be washed to further remove any contaminating C, N, S, or P.

Although an antibiotic subsister was not successfully isolated, one surprising observation from these experiments was how readily microbes with extraordinarily high antibiotic resistances can be isolated from soil, supporting arguments of the soil microbiome as an important origin of antibiotic resistance genes spread among human pathogens [17]. For instance, fosfomycin susceptibility is often defined as occurring at 64 µg mL^{-1} or less [18], whereas up to 1,000 µg

mL^{-1} was used in the enrichments. Fosfomycin-resistant strains were successfully isolated, so future studies could characterize whether these strains exhibit shared or diverse resistance mechanisms.

B. Flowers

HCR-FISH is similar in concept to mono-FISH but with the exception of an additional amplification step in which DNA hairpin structures hybridize to the initiator probes and amplify the fluorophore signal. To test whether the *P. fluorescens* HCR probes can generate any signal at all and whether the chosen fixation procedure has any impact on signal generation, *P. fluorescens* was grown under low and high phosphate conditions, fixed with two different fixation procedures, and, on glass slides, simultaneously probed for the phosphate-regulated genes alkaline phosphatase (AP) and GlpT and, separately, for the constitutively expressed gene LapG. AP and GlpT are oppositely regulated by phosphate, so the relative abundance of these signals was expected to show opposing trends, with relatively greater AP signal under low phosphate and relatively greater GlpT under high phosphate. Since LapG is constitutively expressed, signal was expected to occur in most cells irrespective of phosphate concentration. The results showed that the fixation procedure does not significantly impact signal generation and that while at least some signal could be observed on most slides, it did not follow the expected trends. The low and high phosphate slides probing for AP / GlpT showed little difference in relative signal abundance, and most cells on the LapG slide did not contain signal (Fig. 2). Other problems included intense photobleaching of the GlpT and LapG fluorophores and off-target signal (i.e. not within a DAPI stained cells). Perhaps explaining the off-target signal, it was realized after this initial experiment that a low-stringency buffer was used for the wash steps (20x rather 5x SSCT), so the wash buffers were remade with the recommended SSCT concentration for following experiments.

In the next attempts, lower and higher phosphate conditions (0 and 10 mM rather than 0.1 and 1 mM) were used for the low and high phosphate concentrations, respectively, to test whether these growth conditions could bias signal production towards the expected trends for AP and GlpT. A different gene, LapD, whose probes use a different fluorophore, that is hopefully less susceptible to photobleaching, was chosen to test for constitutive gene expression. As an additional control, a *pst*$^{-}$ mutant, in which the AP gene is always expressed, was also included. While, again, most growth and FISH conditions produced some signal, the AP / GlpT signals did not follow the expected trends, and even with a different flurophore, the signal for the constitutive gene, LapD, did not occur in most cells. However, the greatest success seemed to be gained with the *pst*$^{-}$ mutant. Of the tested probes and conditions, the AP signal for the *pst*$^{-}$ *mutant* seemed brightest and to occur in the greatest proportion of DAPI-stained cells (Figure 3). Somewhat peculiar, off-target signal seemed to occur at higher frequency for the mutant but not in the form of small puncta within large DAPI-stained cells but as cell shapes without corresponding DAP signal.

Finally, *Arabidopsis* plants (Fig. 5) were colonized with *P. fluorescens* and subjected to HCR-FISH. Fortunately, *P. fluorescens* did seem to colonize the plants and even form small biofilms on some root surfaces, as visualized with DAPI (Fig. 4) and with live/dead staining to confirm the presence of viable bacteria. HCR was carried out on plants but, rather than on glass slides, in microcentrifuge tubes. The major problem with these results was the intensity of autoflorescense by the plant, potentially drowning out any HCR signal in bacterial cells. Despite higher stringency washes, off-target signal was again observed in some regions around the plant. Interestingly, other regions around the plant seemed to cause autofluorescence of neighboring bacteria because in these regions, whole bacterial cells appeared as green or red,

whereas on glass slides, when HCR signal is present, it appears as puncta. Future directions should consider optimizing the HCR-FISH slide washing protocol (with the ionic strength lying between the low- and high-stringency washes to maximize the signal) and also an embedding / sectioning or confocal miscopy to better preserve the spatial structure around the root. Mounting the roots for microscopy, especially coverslipping, was particularly disruptive of spatial structure.

C. Metal

Actinomycete strain CS-1 can reduce manganese, as evidenced by clearings it forms on manganese plates. To confirm these clearings are not formed due to a non-reductive process such as acid production, the pH around colonies was measured with a pH electrode and found to be close to neutral. Manganese reduction by CS-1 is likely mediated by a secondary metabolite (e.g. an electron shuttle). Literature on secondary metabolites describes a “phosphate effect” (Whooley & McLoughlin, 1982) where many secondary metabolites, such as pyocyanin in *Pseudomonas aeruginosa*, are upregulated under low phosphate stress. To test whether this holds true for manganese reduction by CS-1, we spotted CS-1 onto manganese plates with increasing concentrations of phosphate. In parallel, we also tested the actinomycete strain RM-1 and *P. fluorescens*. Strikingly, CS-1 manganese reduction was much greater at lower phosphate concentrations. RM-1, however, did not seem to reduce manganese but instead produced a very intense red pigment (data not shown). When *P. fluorescens* was grown as thick biofilms (by spotting cells onto polycarbonate membranes with a high surface tension rather than not directly onto the plate) that likely inhibited oxygen diffusion into the colony, it like CS-1 was found to reduce manganese, but this process did not seem to be regulated by phosphate concentrations under aerobic conditions.

Many manganese reducing bacteria also reduce iron [12], so to test CS-1 for iron reduction and whether it, as predicted by the phosphate effect, does so under low phosphate conditions, potentially releasing adsorbed phosphate back into the environment, the ferric hydroxide ferrihydrite was synthesized, and an attempt was made to adsorb phosphate onto the ferrihydrite, with little success (Fig. 8). Rather than test for phosphate release, CS-1 and *P. fluorescens* supernatants were tested for levels of iron reduction after low and high phosphate growth. Results show that CS-1 does not cause significantly more iron reduction under low phosphate growth (Fig. 9), but interestingly, *P. fluorescens* causes the greatest iron reduction under anaerobic conditions and high phosphate concentrations (or, the opposite of what was expected from the “phosphate effect”). Future directions should investigate the nature of the secreted compound mediating this phosphate-elicited iron reduction by *P. fluorescens*.

IV. Figures

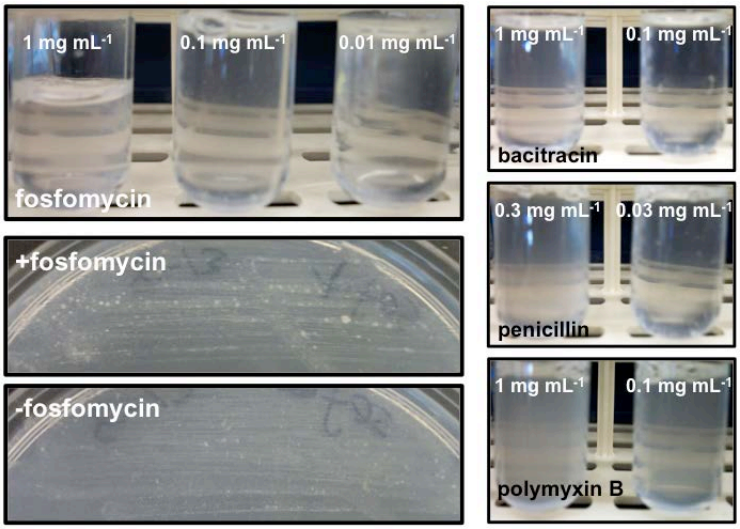

Figure 1. Enrichments for antibiotic users. The left column shows enrichments for users of fosfomycin as a sole phosphorus source. The right column shows enrichments for users of bacitracin, penicillin, or polymyxin B as a sole sulfur source. When the fosfomycin enrichment was struck out onto plates for isolation of single colonies, growth also occurred on a plate where fosfomycin was not included (-fosfomycin), indicating that use of fosfomycin as a sole phosphorus source on the plate including fosfomycin (+fosfomycin) was unlikely.

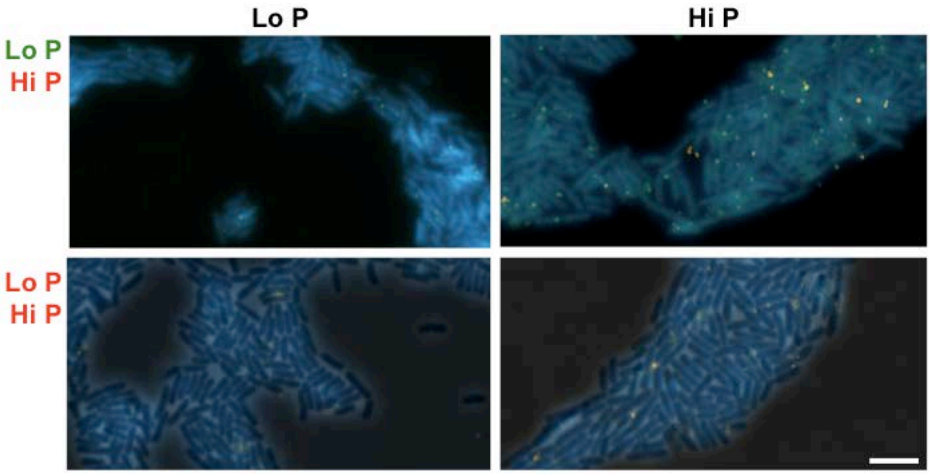

Figure 2. HCR-FISH on the WT. *P. fluorescens* was grown under low (0 mM, left) and high (10 mM, right) phosphate conditions and fixed with 1% PFA. Top row: The relative abundance of the alkaline phosphatase and GlpT signals does not seem to correspond with the phosphate level. Bottom row: Signal for the constitutive LapG gene is infrequent. The transmitted light DIC was overlaid with fluorescence. Green, alkaline phosphatase. Red, GlpT (top) and LapG (bottom). Blue, DAPI. Scale bar, 5 μm.

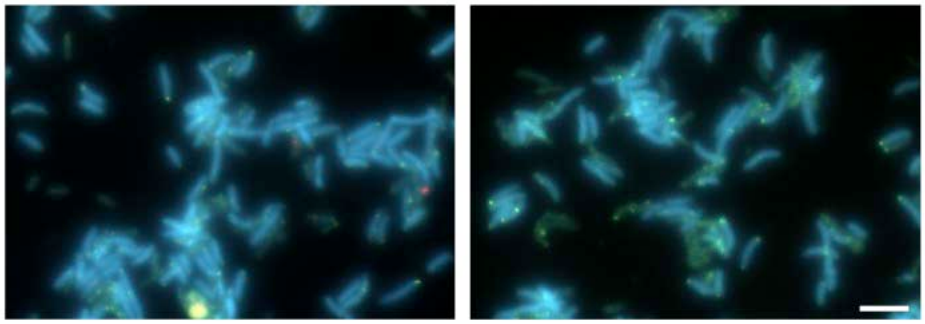

Figure 3. HCR-FISH on the *pst*$^{-}$ mutant. *P. fluorescens pst*$^{-}$ was grown in LB and fixed with 4% PFA. Green, alkaline phosphatase. Red, GlpT. Blue, DAPI. Scale bar, 5 µm.

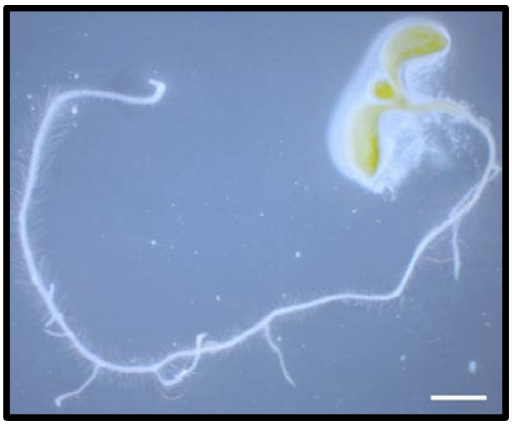

Figure 4. *Arabidopsis thaliana*. Scale bar, 2 cm.

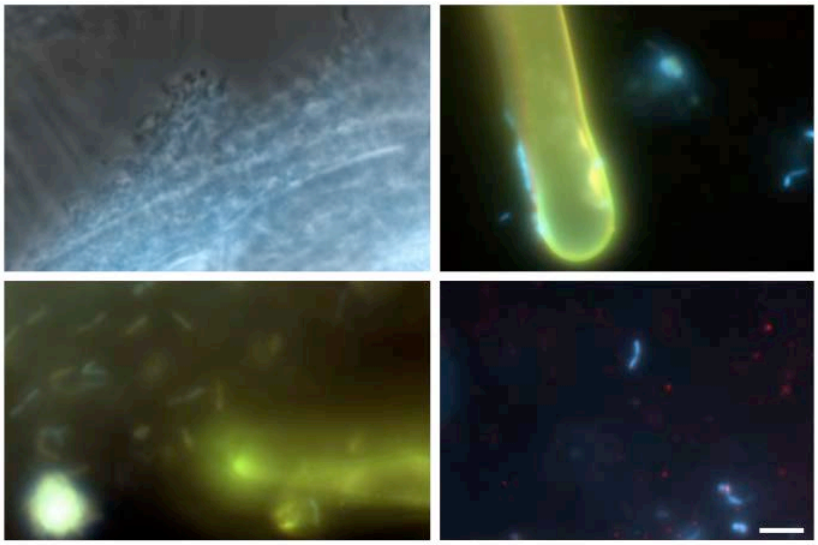

Figure 5. HCR-FISH on *A. thaliana* colonized with *P. fluorescens*. Top left: Overlay of transmitted light DIC with fluorescence. *P. fluorescens* can be found attached to the root surface. Top right: Punctate HCR signal within the DAPI signal was not detected. Bottom left: Root autofluorescence seems to cause higher autofluorescence in the bacterium. Rather than appearing punctate, the green and red HCR signals consume the entire cell. Bottom right: High background and off-target hybridization. HCR signal is located outside cells. Green, alkaline phosphatase. Red, GlpT. Blue, DAPI. Scale bar, 5 µm.

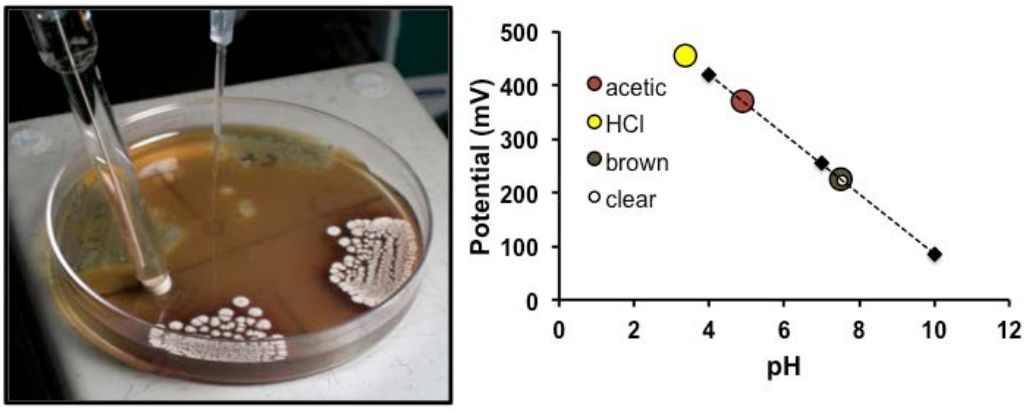

Figure 6. Bacterial manganese reduction is not caused by acid production. Left: The pH of various locations on a manganese plate were measured with a pH electrode. Right: Black diamonds, potentials at 4, 7, and 10. Acetic, location spotted with concentrated acetic acid. HCl, location spotted with concentrated hydrochloric acid. Brown, location not adjacent to bacterial growth. Clear, location adjacent to bacterial growth.

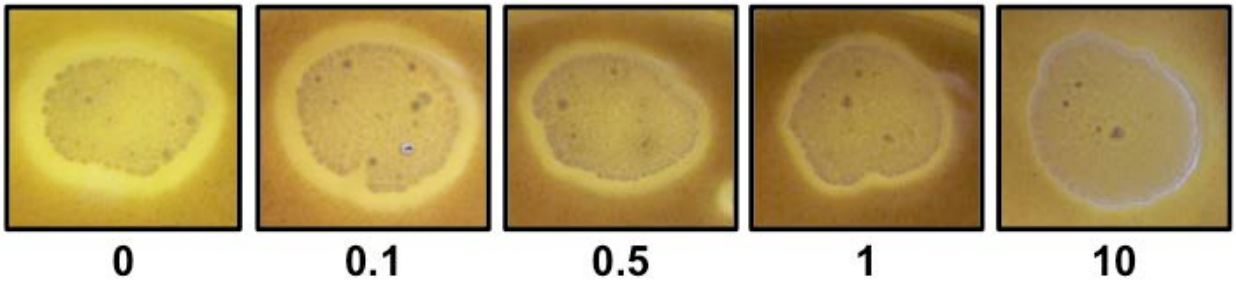

Figure 7. Actinomycete CS-1 reduces manganese in response to phosphate starvation. Numbers indicate the potassium phosphate concentration (mM) in the media.

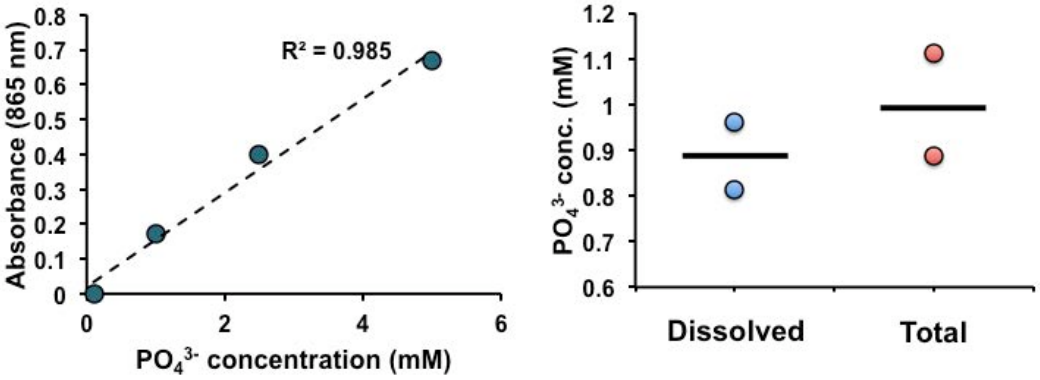

Figure 8. Phosphate was not successfully adsorbed onto ferrihydrite. Phosphate was measured using the molybdenum blue assay (standard curve, left). Dissolved phosphate was measured after filtering the ferrihydrite-phosphate preparation, and total phosphate was measured after fully reducing ferrihydrite-phosphate with hydroxylammonium chloride (right). The difference between total and dissolved phosphate was only ~100 μM, indicating little phosphate was adsorbed onto ferrihydrite.

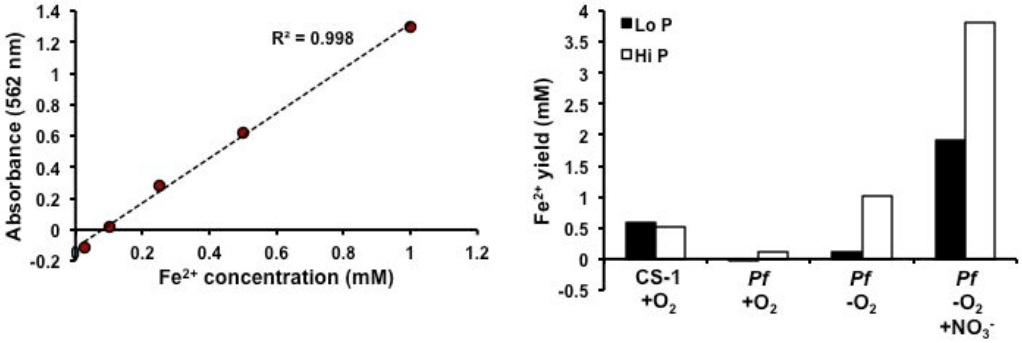

Figure 9. Phosphate abundance rather than starvation triggers *P. fluorescens* iron reduction. Left: standard curve. Right: Strains and growth conditions are indicated on the x-axis and the legend. The y-axis indicates total dissolved and adsorbed ferrous iron measured after incubating culture supernatants with ferrihydrite.

Name	Chemical Formula	M.W.	C		N		S		P	
			mg mL^{-1}	mM (mM $C_4H_4O_4^{2-}$)	mg mL^{-1}	mM (mM NH_4^+)	mg mL^{-1}	mM (mM SO_4^{2-})	mg mL^{-1}	mM (mM PO_4^{3-})
Bacitracin	$C_{66}H_{103}N_{17}O_{16}S$	1422.7	1	0.7 (11.6)	1	0.7 (12.0)	1	0.7 (0.7)		
Fusidic acid	$C_{31}H_{48}O_6$	516.7	1	1.9 (15.0)						
Fosfomycin	$C_3H_7O_4P$	138.1	2	14.5 (10.9)					0.1	0.7 (0.7)
Penicillin	$C_{16}H_{18}N_2O_4S$	334.4	1	3.0 (12.0)	2	6.0 (12.0)	0.33	1.0 (1.0)		
Polymyxin B	$C_{56}H_{100}N_{16}O_{17}S$	1301.6	1	0.8 (10.8)	1	0.8 (12.3)	1	0.8 (0.8)		
Rifamycin	$C_{40}H_{49}NO_{14}$	719.8	1	1.4 (12.9)	10	13.9 (13.9)				
Tetracycline	$C_{22}H_{24}N_2O_8$	444.4	1	2.3 (12.4)	2	4.5 (9.0)				

Table 1. The antibiotics and their concentrations tested in the enrichments. The molar equivalent of succinate, ammonium, sulfate, or phosphate is given in parentheses next to the molar concentration of each antibiotic. Molar equivalence of, for example, succinate was calculated by multiplying the molar concentration of the antibiotic by the ratio of C atoms in the antibiotic to C atoms in succinate. For example for bacitracin, this ratio is 66:4, meaning the molar equivalent of succinate is 66/4 * 0.7, or 11.6 mM succinate. The S enrichments were also tested with antibiotics at 1/10 the concentrations shown in the table. The P enrichment was tested with fosfomycin at 1/10 and 1/100 the concentration shown in the table.

V. References

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