Engineering E. coli for phosphate bioremediation with genes from polyphosphate-accumulating organism Microlunatus phosphovorus

Title:

Engineering *E. coli* for phosphate bioremediation with genes from polyphosphate-accumulating organism *Microlunatus phosphovorus*

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Abstract:

Lakes and coastal regions are increasingly threatened by harmful algal blooms driven by high concentrations of phosphorus, often from domestic and agricultural fertilizers. Algal blooms decrease water quality, interrupt the function of critical infrastructure, and harm businesses reliant on affected bodies of water, disturbing both the environment and the economy. Still, despite the damage that excess phosphorus can cause, phosphorus is a limited resource and a vital nutrient required for agriculture. To improve phosphate management, we developed a strain of modified *Escherichia coli* that can accumulate and store large amounts of phosphorus, while also being able to release stored phosphorus in a controlled manner. As *E. coli* is both robust and easy to engineer, a phosphorus management system utilizing *E. coli* can be used in a wide range of environmental conditions and can be adapted to meet the specific needs of each application scenario and environment. To create our system, we identified genes putatively responsible for phosphate uptake, storage, and preparation for exportation in the polyphosphate-accumulating organism *Microlunatus phosphovorus*. We then transformed these genes into *E. coli* to characterize functions of these previously uncharacterized proteins. Concurrently, we built a bioreactor and designed a suite of cost-effective phosphorus reclamation modules around xerogel-immobilized cells for contained, multipoint phosphate bioremediation. Xerogel beads are a porous glass matrix which entrap cells but allow water, phosphates, and other nutrients to flow through. Characterization of the exopolyphosphatase (PPX2) homolog revealed that PPX2 leads to increased phosphorus release, and preliminary characterization of the polyphosphate kinase 2 (PPK2) homolog C suggests that PPK2 homolog C is potentially responsible for polyphosphate hydrolysis. Through applied genetic, chemical, and mechanical engineering principles we expect to provide a means for preventing harmful algal blooms in both developed and developing countries while also recovering phosphorus for later agricultural use.

Financial disclosure:

Competing interests:

The authors declare that no competing interests exist.

Ethics statement:

N/A

Data availability:

Yes- all data are fully available without restriction. Data can be accessed at http://2016.igem.org/Team:Purdue.

Full submission:

INTRODUCTION

Water phosphates in excess of 25 micrograms per liter are known to drive growth of harmful algal blooms (HABs) during temperatures warmer than 25°C. These blooms compromise water quality by choking oxygen from aquatic ecosystems, and leaching neurotoxins and hepatotoxins into sources of potable water. In doing so, HABs cost global industry more than ten billion USD in damage and threaten human health every year. In addition, there are currently no federal restrictions in the US on water phosphate pollution. Phosphorus is a nonrenewable resource, and in the near future, supply will fail to meet global demands. Therefore, better recycling or reclamation methods must be established (1).

In order to remove excess phosphorus from water sources, a three part system in *Escherichia coli* is envisioned: 1) capture phosphorus from water, 2) store phosphorus as polyphosphate, and 3) release phosphorus from cells on demand. Bacteria are embedded in engineered xerogel beads to prevent their release into the wild and take up luxury amounts of phosphorus, a system adapted from phosphorus accumulating organisms (PAOs), which are currently used in waste treatment plants. These PAOs generate energy by storing phosphate as polyphosphate. ATP dependent and independent polyphosphate kinases bind phosphate into polyphosphate polymers for storage. The genes that normally free phosphates from this chain are downregulated until expressed by a user-defined trigger. The cells then release much of their phosphate and deposit it into the surrounding media. The media can be transported elsewhere while the cells stay embedded in the xerogel beads. This phosphate-enriched media can be further refined to add value to the product. The system offers a wide range of applications including methane digesters, tile drainage systems, wastewater treatment plants, home bioreactors, and floating phosphorus collector units.

Microlunatus phosphovorus is a gram-positive, aerobic, coccus-shaped, actinobacteria of the relatively new genus *Microlunatus*. First isolated in 1995 from activated sludge in the wastewater treatment process, *M. phosphovorus* demonstrates remarkable phosphorus removal from wastewater by being able to take up 48% dry weight in phosphorus alone (2). After Kawakoshi *et al.* sequenced *M. phosphovorus's* genome, phylogenetic analysis revealed the presence of genes that putatively coded for proteins regulating the organism's phosphorus management system: four polyphosphate kinases (PPK), two polyphosphate-dependent glucokinases (PPGK), three phosphate transporters (PiT), and one exopolyphosphatase (PPX) (3) (Fig. 1).
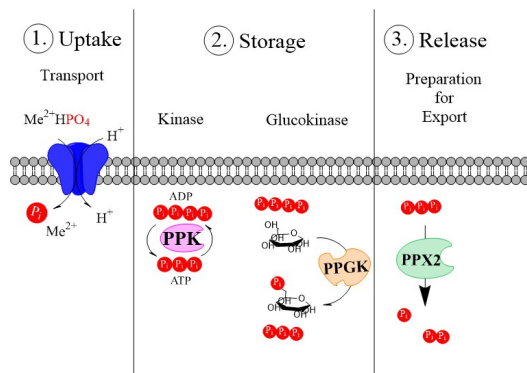

Fig. 1. A diagram depicting the functions of the four phosphorus-related protein families found in *M. phosphovorus* and classifying their function as relating to phosphorus uptake, storage, or release. (PPK = polyphosphate kinase; PPGK = polyphosphate glucokinase; PPX2 = exopolyphophatase)

Polyphosphate kinases catalyze the transfer of polyphosphates between nucleotide mono-, di-, or tri-phosphates and polyphosphate chains, thus leading to the synthesis or hydrolysis of polyphosphate chains. Of the four putative PPKs, one belongs to the subtype PPK1, which favors polyphosphate synthesis using ATP. Two of the putative PPKs belong to the subtype PPK2, which have varying activities for polyphosphate synthesis and hydrolysis. Based on phylogenetic analysis, PPK2 homolog A likely favors polyphosphate synthesis and PPK2 homolog B likely favors polyphosphate hydrolysis. The third PPK, which will be referred to as PPK2 homolog C, has been placed in a phylogenetic cluster consisting of PPKs with underdetermined function, although PPK2 has similarities to both a PPK2 and a polyP-dependent AMP phosphotransferase (PAP), a third PPK subcluster that favors polyphosphate hydrolysis (3).

Comments

> **rcvarga:** "...although PPK2 has similarities to both a PPK2 and a polyP-dependent AMP phosphotransferase (PAP), a third PPK subcluster that favors polyphosphate hydrolysis" I am a bit confused, is a comparison being made of PPK2 to itself?
>> **caleighroleck:** Thanks for the feedback! We were referring strictly to the PPK2-like protein known as PPK2 homolog C, and have updated this sentence for clarification. The sentence now reads "The third PPK, which will be referred to as PPK2 homolog C, has been placed in a phylogenetic cluster consisting of PPKs with underdetermined function, although this PPK known as PPK2 homolog C has similarities to both a PPK2 and a polyP-dependent AMP phosphotransferase (PAP), a third PPK subcluster that favors polyphosphate hydrolysis ."

Polyphosphate glucokinases phosphorylate glucose to glucose-6-phosphate using available polyphophate or ATP. Of the two PPGK homologs present in *M. phosphovorus*, one has been previously characterized; a team from Hiroshima University found that this PPGK can only phosphorylate glucose with polyphosphate and cannot use ATP, (2), thus this homolog will be referred to as PPGK ATP-independent (PPGK ATPI). It is unknown whether the second PPGK homolog is able to use ATP. The inorganic phosphate transporters (Pit) actively transfer inorganic phosphorus in and out of the cell using a proton gradient. These PPK, PPGK, and Pit genes putatively play critical roles in polyphosphate accumulation in *M. phosphovorus* by aiding in the phosphate transport and polyphosphate construction. *M. phosphovorus* also seems to only express one exopolyphosphatase (ppx) gene rather than the two that are typically observed in other Actinobacteria (3). PPX can be utilized for its ability to hydrolyze terminal phosphates of polyphosphate chains in order to minimize the overall amount of orthophosphate leaving from the system. (Fig. 1.) While this organism holds significant promise for application in enhanced biological phosphorus removal (EBPR), not enough characterization has been done with it to engineer an optimized, robust PAO.

Comments

> **rcvarga:** "Polyphosphate glucokinases phosphorylate glucose to glucose-6-phosphate using available polyphophate or ATP." Change "polyphophate" at the end of the sentence to "polyphosphate"! :)
> **caleighroleck:** Thank you, this has been changed.

In order for the implementation of a synthetic biology solution to be feasible, an effective system of containment to prevent organisms from escaping into the environment must be developed. For example, if microorganisms capable of accumulating large quantities of phosphorus were to grow unchecked in an ecosystem, plants and animals may perish from lack of this essential nutrient. In the context of this project, modified *E. coli* were incorporated into xerogel silica beads created using the sol-gel method. These beads,

several millimeters in diameter, encapsulated microorganisms within a porous matrix that allowed water, phosphorus, and other nutrients to flow through but immobilized bacteria, effectively containing them and restricting their growth.

The combination of biological construct and physical mechanism compose a novel system that could effectively remove phosphorus from water, store it for later use, and recycle this nutrient by releasing it on demand. Through assembling a genetic circuit using standard synthetic biology procedures and iGEM materials and protocols, measuring the efficacy of the modified organism, and creating structures to contain and support the system, a solution to the global rise in the growth of HABs is proposed.

MATERIALS AND METHODS

Plasmid assembly and transformations. We ordered all genes as gBlocks (Integrated DNA Technologies, Coralville, IA), using the GenBank sequence for each of the ten genes except PPK2 homolog C, for which we used the NCBI Reference Sequence. (Table 1). The sequences for each gene, as well as their putative function, was determined by Kawakoshi *et al* in 2012 (3).

Table 1: Sequence Accession Information for Genes

Gene	Sequence Accession Number	BioBrick Registry Number
Pit homolog A	BAK33067.1	BBa_K1988002
Pit homolog B	BAK35997.1	BBa_K1988004
Pit homolog C	BAK38120.1	BBa_K1988006
PPK1 homolog	BAK37784.1	BBa_K1988010
PPK2 homolog A	BAK3589.1	BBa_K1988014
PPK2 homolog B	BAK38044.1	BBa_K1988016
PPK2 homolog C	WP_041792057.1	BBa_K1988018
PPGK homolog	BAK35675.1	BBa_K1988008
PPGK ATPI	BAK33557.1	BBa_K1988010
PPX2 homolog	BAK37491.1	BBa_K1988000

With the exception of PPK1 homolog, we ordered all genes as a single gBlock. All gBlock segments began with a HindIII restriction site at the 3' end, followed with the weak, constitutive Anderson promoter BBa_J23015, the standard BioBrick prefix, containing an EcoRI and an XbaI restriction site, and the ribosome binding site BBa_J34803. The 10X polyhistidine tag BBa_K844000 was fused to the c-terminus of every protein, with the exception of PPK2 homolog A, which had a 6X polyhistidine tag fused to the n-terminus due to synthesizing complexities. We split the two gBlocks for PPK1 homolog in the middle of the gene. Following each gene, the gBlocks had the standard BioBrick suffix, containing a SpeI and Pst1 restriction site, the terminator BBa_B0010, and then a HindIII restriction site at the 5' end.

After we received the gBlocks, we amplified them via PCR. We designed the forward primer (Thermo Fisher Scientific, Waltham, MA) using the sequence for the HindIII restriction site and the first 18 bases of the Anderson promoter and adding the bases "ctat" prior to the HindIII restriction site (ctataagcttttacggctagctcagtc). We designed the reverse primer (Thermo Fisher Scientific, Waltham, MA) using the complementary sequence for the HindIII restriction site and the last 14 bases of the terminator and adding the bases "atat" prior to the restriction site (atataagcttgagagcgttcaccg). Gibson assembly was used to join together the two PPK1 homolog gBlocks, as described in Nature Methods (4). Then, we performed 3A assembly to ligate each gene and the medium strength constitutive Anderson promoter BBa_J23016 into the plasmid PSB1C3 or PSB1A3. 3A assembly was performed using the standard iGEM protocol (5). We transformed the plasmid into NEB 5-alpha Competent *E. coli* in accordance with NEB's recommended protocol (6) and plated the transformants on LB agar plates with either chloramphenicol (for PSB1C3 plasmids) or ampicillin (for PSB1A3 plasmids), producing twelve different strains (Table 2). Colonies were inoculated and mini-prepped. We submitted the plasmids to the Purdue Genomics Core Facility to validate the sequence via Sanger sequencing.

Table 2: *E. coli* strains

Strain Name	Gene	Plasmid
cPitA	Pit homolog A	PSB1C3
cPitB	Pit homolog B	PSB1C3
cPitC	Pit homolog C	PSB1C3
cPPGK	PPGK homolog	PSB1C3
cPPGK-ATPI	PPGK ATPI	PSB1C3
cPPK1	PPK1 homolog	PSB1C3
cPPK2A	PPK2 homolog A	PSB1C3
cPPK2B	PPK2 homolog B	PSB1C3
aPPK2C	PPK2 homolog C	PSB1A3
cPPK2C	PPK2 homolog C	PSB1C3
aPPX2	PPX2 homolog	PSB1A3
cPPX2	PPX2 homolog	PSB1C3

Analysis of phosphorus uptake, storage, and release. We inoculated strains of *E. coli* containing genetic constructs theorized to lead to increased phosphorus uptake or increased phosphorus exportation in LB broth. The culture was then diluted to produce 250 mL of a minimal media culture with an optical density of 0.01 at 600 nm. After inoculation, we took 5 mL samples hourly for 6 hours, starting with hour 0. We froze all samples at −20 °C for later analysis. We collected an additional sample at 72 hours. After sample collection, we centrifuged the samples to separate the cellular component from the supernatant and transferred the supernatant to a second tube. The total phosphorus content of the supernatant was quantified using an Inductively Coupled Plasma (ICP) spectrometer from the United States Department of Agriculture National Soil Erosion Research Lab.

In addition, we inoculated all strains in 5 mL of LB broth supplemented with K_2HPO_4 to produce a 2 mM PO_4^{3-} solution, which is deemed a sufficient media concentration to assess phosphorus accumulation (7). We then extracted polyphosphate granules and quantified polyphosphate concentration spectrophotometrically with a toluidine blue and acetic acid solution, in accordance with the Mukherjee and Ray protocol (8). The absorbance of each sample at 580 nm was compared against a standard curve prepared using sodium phosphate glass type 45.

Production of silica beads. We formed silica beads as a means of immobilizing bacteria. The sol-gel process combines an acidic hydrolyzed solution of tetramethyl orthosilicate (TMOS) with *E. coli* suspended in neutral phosphate-buffered saline (PBS), as described in Rickus *et al* (8). When the TMOS sol-precursor is added to the buffer, the pH increase favors rapid polymerization of sol-gel at room temperature. To form the silica beads, we dripped the TMOS sol-precursor and PBS mixture into a vat of mineral oil using a syringe.

To verify that the silica beads can effectively immobilize *E.coli*, we produced a red fluorescent protein-expressing strain of *E. coli* using the BioBrick construct BBa_J04450 (a LacI regulated promoter, ribozyme binding site, an engineered Discosoma striata red fluorescent protein mutant, and a double terminator) in the plasmid PSB1C3. We then added this strain to the PBS, which was used in the production of silica beads. The beads were then imaged with the EVOS Cell Imaging System in order to detect red fluorescence both within and between the beads.

Bioreactor construction and iterative design. Seeking a solution that could be implemented in a variety of different scenarios, we consulted public and private stakeholders to determine a list of design specifications with quantitative metrics for a desirable physical prototype, such as a cost of less than $100 USD to construct, $25 to maintain annually, and a water phosphorus concentration less than 0.025 ppm post-filtration. In collaboration with the United States Department of Agriculture National Soil Erosion Research Laboratory (USDA-NSERL), we constructed a bioreactor to allow water to flow over and through silica beads, emerging with a lesser concentration of phosphorus after passing through the system. A central goal of this design was to produce a prototype that could, with a few modifications, be practical in many environments, including agricultural field tile drains, within wastewater treatment facilities, in septic tank water treatment systems, and below city streets in sewer drains.

Comments

rcvarga: " Seeking a solution that could be implemented in a variety of different scenarios, we consulted public and private stakeholders to determine a list of design specifications with quantitative metrics for a desirable physical prototype, such as a cost of less than $100 USD to construct, $25 to maintain annually, and a water phosphorus concentration less than 0.025 ppm post-filtration" This could be moved to the discussion or acknowledgements section if so desired

caleighroleck: While we appreciate this suggestion, we ultimately decided to leave this section in the materials and methods section. We wanted to include our bioreactor design process with our methods, and these discussions and the design criteria which they produced heavily influenced bioreactor design.

Two five-gallon buckets--one "reactor" and one "reservoir"--are connected by one-inch flexible tubing and an aquarium pump powered by an electrical outlet to keep the water level stable within the reactor bucket (Fig. 2). Three exit ports around the base of the reactor bucket allow gravity-driven effluent to flow through an additional length of tubing into one of three water filter canisters. Each canister, or phosphorus reclamation unit, contains an inner water filter; between the water filter and outer canister wall, silica beads containing modified microorganisms are packed. Water to be treated would enter the reservoir bucket, be pumped into the reactor bucket, exit the reactor bucket through its base, then flow through a filter canister and bead matrix where its phosphorus concentration is reduced before leaving the system via the outflow spigot (Fig. 2).
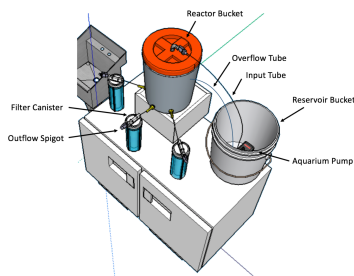

Fig. 2: A bioreactor prototype utilizes two five-gallon buckets, an aquarium pump, and water filter canisters to contain silica beads encapsulating modified *E. coli*.

A theoretical design iteration includes the replacement of the filter canisters with a bead-packed pipe. This pipe would decrease water velocity as its diameter decreases, enabling maximum phosphorus uptake by bacteria within the beads. Diameter could be adjusted to optimize the volume of water capable of passing through the system and the amount of phosphorus that could be absorbed from it.

RESULTS AND DISCUSSION

Plasmid assembly and transformations. Sequencing results confirmed that only the transformants for the strains aPPK2C, aPPX2, and cPPX2 contained the correct insert. Sequencing results for all other strains indicated that colonies only contained the PSB1C3 plasmid without the correct insert, and so the plasmid self-ligated. In future experimentation, we plan to treat the plasmid with phosphatase to remove the 5' phosphate and prevent self-ligation of the backbone when assembling the plasmids for the other strains.

Analysis of phosphorus uptake, storage, and release. We inoculated the strain aPPX2 and an unmodified strain of *E. coli* in a minimal media and allowed to grow for 72 hours. After centrifuging the samples and analyzing the total phosphorus content of the supernatant with an Inductively Coupled Plasma spectrometer, we compared the total phosphorus concentration on both supernatants. We expected the phosphorus concentration of aPPX2's supernatant to be greater than that of unmodified *E. coli*, as PPX2 cleaves the terminal phosphate from a polyphosphate chain, thus preparing an orthophosphate molecule for exportation. Therefore, since aPPX2 contains a putative PPX2, and the unmodified strain does not, aPPX2 should theoretically release more phosphorus into the supernatant than unmodified *E. coli*. The concentrations of total phosphorus in the supernatant were determined to be 2.412 ppm and 0.741 ppm, respectively, and so, as expected, the supernatant for aPPX2 had a greater total phosphorus than that of unmodified *E. coli* and the PPX2 homolog is behaving as expected.. (Fig. 3). A student's t-test indicated that PPX2-producing *E. coli* had significantly more ($p < 0.005$) phosphorus in the supernatant than that of unmodified *E. coli*. The other sequence verified strains, aPPK2C and cPPX2, were not analyzed in this manner, as cPPX2 contains the same gene as aPPX2, and aPPK2 contains the gene for a putative polyphosphate kinase, which would not produce a change in phosphorus uptake or exportation, only polyphosphate storage.

Comments

rcvarga: "We inoculated the strain aPPX2 and an unmodified strain of E. coli in a minimal media and allowed to grow for 72 hours." Either "in minimal media" or "in a minimal medium." Media is plural

caleighroleck: Thank you! This has been changed.

rcvarga: "A student's t-test" Not necessary to say "a student's" T-test, just a T-test would be sufficient.

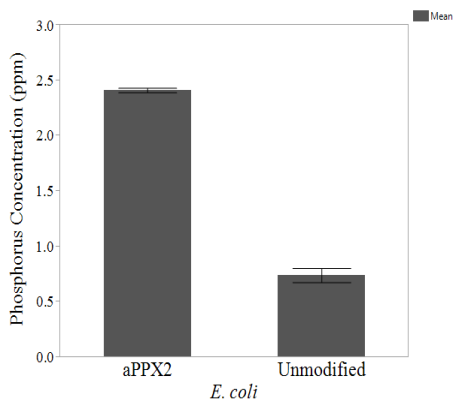**caleighroleck:** Thank you! This has been changed.

Fig. 3. The concentration of total phosphorus present in the the supernatant after 72 hours of growth for the strains aPPX2 and unmodified *E. coli*. Bars represent standard error.

> **Comments**
>
> **VeroniqueKiermer:** Could you provide the sample size (are these multiple measurements on the same culture or on multiple supernatants?), and ideally the data underlying the graph to allow the reader to appreciate the distribution. thanks!
>> **caleighroleck:** Thank you for your feedback! The sample size is 2 each, and these come from two different supernatants. We will include the sample size in the figure legend. Additionally, while we do not plan on including the raw data in the final report for the sake of keeping the paper concise, the underlying data can be accessed at https://osf.io/snrgg/ in the file Phosphorus Uptake Assay Supernatant Concentration.

We used a standard curve to estimate the intracellular polyphosphate concentration of several strains of *E. coli* . The standard curve compares absorbance at 630 nm to polyphosphate concentration, and had an R^2 value of 0.975 and a correlation of −0.987 (Fig. 4). As this is a strong correlation, a linear model was concluded to be a reasonable way to estimate intracellular polyphosphate concentration colorimetrically.
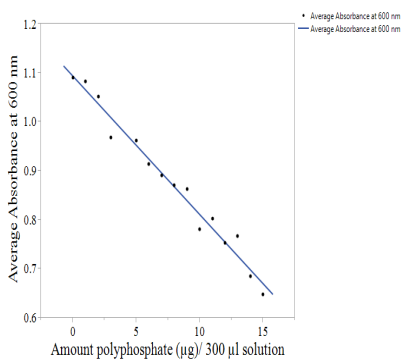

Fig. 4 The standard curve prepared for quantifying intracellular polyphosphate colorimetrically. The R^2 value is 0.975 and the line of best fit is Y=1.094-0.02895X.

The amount of intracellular polyphosphate was then estimated for four strains of *E. coli*: aPPK2C, aPPX2, cPPX2, and an unmodified strain. We hypothesize that both aPPX2 and cPPX2 should have less intracellular polyphosphate than unmodified *E. coli*, as both contain a putative PPX2, which hydrolyzes polyphosphate. Given the current knowledge on aPPK2C, we hypothesize that the intracellular polyphosphate concentration for aPPK2C would be different than that of unmodified *E. coli*, but we cannot predict whether the concentration would be higher or lower, as PPK2 homolog C's activity for polyphosphate synthesis or hydrolysis could not be predicted based on phylogenetic analysis (3). All three modified strains were estimated to have a lower concentration of intracellular polyphosphate in comparison with unmodified *E. coli* (Fig. 5). Still, pairwise t-tests did not detect any statistical significance with an α-level of 0.05. When compared to the unmodified strain, aPPK2C had a p-value of 0.3969, aPPX2 had a p-value of 0.3029, and cPPX2 had a p-value of 0.5433. Still, as we only obtained data for three samples for each strain, the lack of statistical significance could potentially be due to low sample size.

> **Comments**
>
> **rcvarga:** "we hypothesize that the intracellular polyphosphate concentration for aPPK2C would be different than that of unmodified E. coli," I would reword this; generally hypotheses are included in the introduction, not the results or discussion.
>> **caleighroleck:** While we appreciate the feedback, we are deciding to keep this specific hypothesis in the results and discussion section, as this is not our central hypothesis, but rather a hypothesis for this specific assay. Therefore, we believe this hypothesis is important for understanding these specific results, but not our whole project.
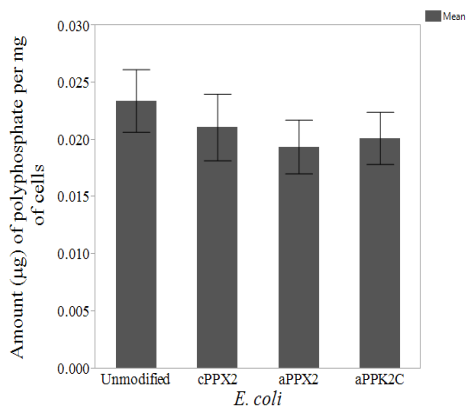
Fig. 5. The average intracellular polyphosphate concentration of several strains of *E. coli.* The bars represent standard error.

The results suggest the function of the putative PPX2 homolog as an exopolyphosphatase, as PPX2 homolog expression in *E. coli* increased phosphate release, and may lower intracellualr polyphosphate concentration in *E. coli,* although further testing is needing. This PPX2 homolog was previously uncharacterized in both *M. phosphovorus* and *E. coli.* Despite M. phosphovorus being gram-positive and *E. coli* being gram-negative, the PPX2 homolog should still produce a functional protein because PPX2 is not an integral membrane protein. While exopolyphosphatase leads to the expulsion of phosphorus from the cell, PPX2 does this by preparing intracellular phosphorus for expulsion through the hydrolysis of polyphosphate to produce orthophosphate. Therefore, differences in cell wall and membrane structure should not affect the ability of *E. coli* to produce a functional exopolyphosphatase native to *E. coli.*

Due to the lack of statistical significance for results for strains containing PPK2 homolog C, we cannot confirm the function of PPK2 homolog C, although results suggest that PPK2 homolog C expression may decrease the amount of intracellular polyphosphate. Further testing is needed. Still, despite the levels of uncertainty associated with these results, these results are the first indication of the activity for PPK2 homolog C for polyphosphate synthesis or hydrolysis. Not only was PPK2 homolog C previously uncharacterized in both *M. phosphovorus* and *E. coli,* but phylogenetic analysis placed PPK2 homolog C in a distinct cluster of putative PPK2 homologs with undetermined functions. Still, while PPK2 homolog C belongs to this distinct cluster of polyphosphate kinases, PPK2 homolog C is similar to both a PPK2 and a polyP-dependent AMP phosphotransferase (PAP), another polyphosphate kinase subtype. While the PPK2 polyphosphate kinase subfamily contains PPKs with activities as either a polyphosphate synthase or hydrolase, PAPs have an affinity for polyphosphate hydrolysis (3). There, these preliminary results and PPK2 homolog C's similarity to a PAP as well as a PPK2 have caused us to form the hypothesis that PPK2 homolog C has a polyphosphate hydrolase activity. Once again, despite the differences in cell wall and membrane structure, *E. coli* should be able to produce a functional PPK from *M. phosphovorus,* as PPK, as PPK2 homolog C is not an integral membrane protein. *M. phosphovorus* does contain four PPK homologs though, and while we were interested in all homologs, we only studied PPK2 homolog C, as aPPK2C was the only strain containing a sequence-verified plasmid with a PPK homolog.

Production of silica beads. After producing a strain of *E. coli* expressing a red fluorescent protein, immobilizing the strain within silica beads, and producing silica beads using the same method, but without adding *E. coli,* we viewed both samples of silica beads with an EVOS Cell Imaging System to analyze red fluorescence. The beads containing red fluorescent protein (RFP)-expressing *E. coli* displayed red fluorescence, while the negative control beads that did not contain *E. coli* did not exhibit red fluorescence. (Fig. 6.) As the red fluorescence was present within silica beads produced to contain red-fluorescent E. coli, these results indicate that the silica beads successfully immobilized the *E. coli.* If the beads do not contain immobilized *E. coli,* they would not exhibit fluorescence, just as the silica beads produced without *E. coli* did not exhibit fluorescence. Additionally, we did not detect red fluorescence between the beads for the sample of beads that contained RFP-expressing *E. coli.* This absence of fluorescence preliminarily indicates that the *E. coli* is not leaching out of the beads, as, if red-fluorescent *E. coli* leached out of the silica beads, red fluorescence outside of the beads would be detected. Still, in the future, more extensive leaching assays will be conducted. Additionally, while previous studies have shown that bacteria have 40% cell viability after one month of encapsulation in silica beads (10), we plan on studying the viability of our own strain of polyphosphate-accumulating *E. coli* in the future.

Comments

VeroniqueKiermer: I think you are touching on two very important points here, as leaching or poor cell viability could impact implementation. These definitely merit more investigation. Perhaps you could discuss some mitigation strategies too.

caleighroleck: In response to your suggest, we have added two different mitigation strategies to address both issues. For cell viability, these include vacuum evaporating the alcohol byproduct that forms from the gel polymerization and clumping cells. For bacterial leaching, this also includes the cell clumping solution, as well as engineering the cells to be dependent on an additive to the silica beads. Thank you for your feedback.
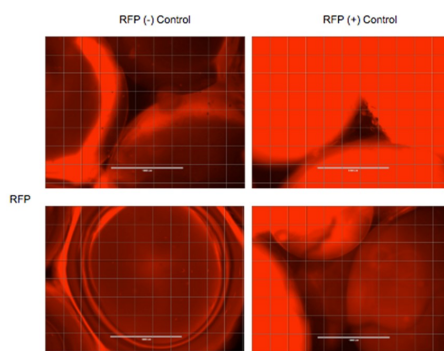

Fig. 6. The silica beads tested for red fluorescence. On the left: Silica beads without *E.coli*. On the right: Silica beads with red fluorescent protein-producing *E. coli.*

CONCLUSION

Due to the flexibility of this technology, future improvements and additional application scenarios are limited only by imagination. After adding an inducible promoter to the genetic construct within *E. coli* governing phosphorus exportation, the system would be able to store phosphorus within it to be released after the promoter is triggered. A kill switch could also be added to improve containment efficacy, decreasing the probability that no modified organism could disrupt the environment. Continued iterations of the bioreactor would enable further customization for different applications; for example, increasing the size of the reservoir bucket and the number of exit ports from the reactor bucket would increase the volume of water that could pass through the system. The addition of other genes such as those related to nitrogen uptake, pesticide digestion, and pharmaceutical breakdown would expand the number of pollutants capable of being captured and reclaimed by the bioreactor system. After adding these genes, the entire genetic construct could be integrated into the *E. coli* genome, making the organism a truly synthetic solution with immense positive societal and industrial impact.

Within the scope of a summer, genes from recently-sequenced *M. phosphovorus* genome were characterized individually, namely PPX2 homolog and PPK2 homolog C, and novel combinations imagined, revealing behaviors mirroring predictions given observed homologies. The complementary bucket bioreactor demonstrated its ability to effectively circulate water, indicating the possibility for future diverse applications through continued iteration.

ACKNOWLEDGEMENTS

LITERATURE CITED

> **Comments**
>
> **jgoupil:** Nice work! I believe PLOS uses "Vancouver" style (outlined by ICMJE) for their references. If you would like to publish your work, you will need to adapt these references to Vancouver style
> **caleighroleck:** Thank you for your feedback! This has been changed.

1. R.M. Kudela et al. (2015). Harmful Algal Blooms. A Scientific Summary for Policy Makers. IOC/UNESCO, Paris (IOC/INF-1320).

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> **Comments**
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> **PLOSFeedback:** Authors used genes from a phosphorous removing bacterium and cloned them into E.coli. They then built a bioreactor that contained a mesh to trap the bacteria but allow water/ phosphorous to flow through, with the idea of containing transgenic animals if they were to be used in lakes. Overall this is well reported but possibly too preliminary a report to be submitted in full to PLOS ONE as it currently stands. But it could certainly be submitted as an iGEM report on PLOS Collections, and it will be included in the PLOS iGEM 2016 Collection. If we can provide any clarifications or support please do let us know - collections@plos.org
> **caleighroleck:** Yes, our plan was to submit this as an iGEM Report and not a full PLOS ONE article.Thank you for the feedback!