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Identification of extracellular glycerophosphodiesterases in *Pseudomonas* and their role in soil organic phosphorus remineralisation

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In soils, phosphorus (P) exists in numerous organic and inorganic forms. However, plants can only acquire inorganic orthophosphate (Pi), meaning global crop production is frequently limited by P availability. To overcome this problem, rock phosphate fertilisers are heavily applied, often with negative environmental and socio-economic consequences. The organic P fraction of soil contains phospholipids that are rapidly degraded resulting in the release of bioavailable Pi. However, the mechanisms behind this process remain unknown. We identified and experimentally confirmed the function of two secreted glycerolphosphodiesterases, GlpQI and GlpQII, found in *Pseudomonas stutzeri* DSM4166 and *Pseudomonas fluorescens* SBW25, respectively. A series of co-cultivation experiments revealed that in these *Pseudomonas* strains, cleavage of glycerolphosphorylcholine and its breakdown product G3P occurs extracellularly allowing other bacteria to benefit from this metabolism. Analyses of metagenomic and metatranscriptomic datasets revealed that this trait is widespread among soil bacteria with *Actinobacteria* and *Proteobacteria*, specifically *Betaproteobacteria* and *Gammaproteobacteria*, the likely major players.

Although many forms of phosphorus (P) exist in soils, plants can only acquire simple inorganic orthophosphate (Pi) and therefore P scarcity often limits plant growth and hence crop production\(^1\). To alleviate P scarcity in soils, rock phosphate is heavily applied to many agricultural cropping systems. However, this application results in several negative environmental and geo-political consequences\(^2,3\). In addition to the secretion of organic acids that aid in the release of Pi chelated to various metal ions, plants also secrete an array of enzymes to facilitate the breakdown of various forms of complex P\(^5,7\). However, soil microorganisms also play a key role in both the solubilisation of Pi minerals and the remineralisation of organic forms of P and may help reduce our dependency on rock phosphate as a fertiliser\(^8,9\). In soils, organic P often represents 30–65% of total P\(^10\), the majority of which are simple phosphomonoesters and phosphodiesters (up to 90%)\(^11\), or phytate (up to 50%)\(^9\). The organic P content of soil also contains phospholipids of both microbial and plant origin that are rapidly degraded resulting in the release of bioavailable Pi\(^12,14\). Furthermore, the addition of phospholipids to soil results in an increase in plant (Barley) P uptake\(^15\). However, the key genes and enzymes responsible for this potentially beneficial metabolic step have not been identified in soils.

Various microbial secreted enzymes (exoenzymes) including alkaline phosphatases (ALP), acid phosphatases (ACP), phytases, phosphonatases, nucleases and phosphodiesterases can facilitate the remineralisation of organic P into Pi thus improving soil P fertility\(^16,18\). These exoenzymes are usually secreted in response to Pi scarcity.
and this process, as well as the expression of other proteins, is regulated by a two component regulatory system, consisting of a DNA-binding transcriptional regulator (PhoB) and transmembrane histidine kinase (PhoR)\(^\text{19}\). In the rhizobacterium *Pseudomonas putida* BIRD-1, mutagenesis of the genes (*phoBR*) encoding PhoBR silenced the normal induction (upon Pi-depletion) of various Pi-scavenging proteins, as well as the high affinity Pi transporter, PtsSABC\(^\text{20}\). This mutant could still grow on Pi due to the presence of a low affinity Pi transporter (PitA), whose expression was not affected by mutation of *phoBR*\(^\text{20}\). ALPs, which are abundantly secreted in response to Pi-depletion\(^\text{20, 21}\), are thought to be promiscuous enzymes for numerous phosphomonoesters and phosphodiester and their abundance and diversity is affected by bioavailable P and other environmental factors\(^\text{22-24}\).

Although ALPs are promiscuous enzymes, they cannot release Pi from glycerophosphodiesterases, such as glycerolphosphorylcholine (GPC), which forms the head group of phospholipids, such as phosphatidylcholine. In this instance, specific glycerolphosphodiester phosphodiesterases (GDPD, EC_3.1.4.46), either secreted (GlpQ) or located in the cytoplasm (UgpQ), are required to release sn-glycerol-3-phosphate (G3P) and the corresponding alcohol\(^\text{27, 28}\). Therefore, the release of Pi from glycerophosphodiesterases is a multi-step process with the final step performed by ALPs. GDPDs, namely GlpQ, produced by phylogenetically distinct bacteria such as *Escherichia*, *Bacillus*, *Streptococcus*, *Mycoplasma* and *Streptomyces* are often required for establishing various relationships (pathogenic or mutualistic) between bacteria and their recipient hosts\(^\text{21, 27, 29, 30}\). A periplasmic GlpQ was first characterised in *E. coli*, with the encoding gene (glpQ) co-located with glpT, the latter encoding a G3P transporter\(^\text{21}\). Interestingly, in *E. coli* the *ugpBAECQ* operon, which encodes a cytosolic G3P and a corresponding ABC transporter for GPC, is induced by Pi depletion, whereas glpQT is part of the glycerol regulon\(^\text{27, 28}\). In contrast, in *B. subtilis* the glpQT operon is induced by Pi-depletion as well as by glycerol, but is repressed in the presence of a more favourable carbon source\(^\text{31}\). *Pseudomonas stutzeri* DSM4166, a nitrogen-fixing bacterium isolated from the rhizosphere of yellow indiangrass (*Sorghum nutans*)\(^\text{32}\), secretes numerous proteins in response to Pi-depletion\(^\text{33}\). The most abundant proteins found in the exoproteome of Pi-depleted *P. stutzeri* DSM4166 include ALPs, nucleotidases and a putative GDPD\(^\text{34}\). This putative GDPD contains the Pfam domain 03009, encompassing the GDPD family, found in both GlpQs produced by *E. coli* and *B. subtilis*. To date, the contribution of GlpQ to phospholipid transformations in soils is unknown.

Here, we determined whether the potential GlpQ from *P. stutzeri* DSM4166 is capable of converting GPC to G3P. We also identified and experimentally confirmed another GlpQ-type enzyme found in *P. fluorescens* SBW25. Furthermore, we also examined whether G3P was cleaved extracellularly releasing exogenous Pi. The consequence of this metabolism with respect to organic P cycling was investigated in a series of *Pseudomonas* co-cultivation experiments. Finally, we analysed *in silico* the distribution and transcription of GlpQ and other GDPD enzymes in various soil/rhizosphere systems. Together, our results present a mechanism for the release of Pi from phospholipid degradation in soil.

**Results**

**Distribution of GlpQ-like homologs in *Pseudomonas* strains.** Results from a previous exoproteomics experiment\(^\text{35}\) revealed that *P. stutzeri* DSM4166 secreted an exoprotein abundantly (encoded by PSTAA_4169) in response to low Pi availability. PSTAA_4169 is predicted to encode the GDPD domain (Pfam 03009) and showed homology to the periplasmic glycerolphosphodiesterase GlpQ found in *E. coli* (identity 36.78%, e-value, 2.0 e-54). PSTAA_4169, hereafter termed glpQ, encodes a protease (GlpQI) containing all the conserved residues associated with characterised GDPDs\(^\text{35}\) (Fig. S1). Thus, it was hypothesised that this Pi-responsive exoprotein is capable of cleaving the diester (C-O-P-O) bond associated with the headgroup of phospholipids. GlpQI was 50 amino acids longer than GlpQ homologs from other *Pseudomonas* strains and the cleavage site for the signal peptide of this protein is predicted to be located between amino acid residues 78–79. Furthermore, no peptides before amino acids longer than GlpQ homologs from other *Pseudomonas* strains encode the genes for both GlpQI and GlpQII. *P. putida* SBW25 with GPC as the sole source of P (100 μM) (Fig. 2). We also grew *P. putida* BIRD-1 on GPC as the sole source of P as this bacterium does not encode GlpQI or GlpQII in its genome.

To better understand the phylogeny of GDPD-like proteins, a number of *Pseudomonas* strains as well as various phylogenetically distinct bacteria (e.g. *Mycoplasma pneumoniae*, *Streptococcus pneumoniae*, *Haemophilus influenzae*), whose genomes have been deposited in the Integrated Microbial Genomes database at the Joint Genome Institute (IMG/IGI) and are known to harbour genes encoding previously characterised GlpQ homologs (Table S5), were screened for the presence of genes encoding enzymes containing the Pfam domain 03009. The characterised UgpQ from *E. coli*\(^\text{36}\) and other cytoplasmic GDPDs (putative and characterised) were retrieved using this method. The various GDPDs clustered into several groups, including one group that possessed homologs of GlpQI (Fig. 1). In this group, homologs were predominantly retrieved from various *P. stutzeri* strains with no homologs of GlpQI found in the majority of other well-known *Pseudomonas* species e.g. *P. putida*, *P. fluorescens*, or *P. syringae*. However, we also identified another cluster (termed GlpQII in Fig. 1) that contained several sequences also predicted to possess signal peptides. One of these sequences, retrieved from *P. fluorescens* SBW25 (ORF PFLU4789, hereafter referred to as GlpQII), showed similarity to GlpQI (identity 28.77%, e-value, 4.0 e-23). The GlpQII cluster also contained sequences retrieved from other *Pseudomonas* spp. Like GlpQI produced by *P. stutzeri*, GlpQII also contained the conserved residues required for GDPD activity. Interestingly, the genomes of *P. aeruginosa* strains encode the genes for both GlpQI and GlpQII.

**GlpQ is essential for growth on glycerolphosphodiester in *Pseudomonas***. To test the hypothesis that these *Pseudomonas* GlpQ-like homologs have a role in growth on phospholipid headgroups, we cultured *P. stutzeri* DSM4166 and *P. fluorescens* SBW25 with GPC as the sole source of P (100 μM) (Fig. 2). We also grew *P. putida* BIRD-1 on GPC as the sole source of P as this bacterium does not encode GlpQI or GlpQII in its genome. The wild type (WT) strains of *P. stutzeri* DSM4166 and *P. fluorescens* SBW25 grew on the phosphodiester GPC whilst *P. putida* BIRD-1 did not, suggesting that GlpQI and GlpQII can function as a GDPD (Fig. 2). All three
Figure 1. Phylogenetic analysis of GlpQI (DSM4166) and GlpQII (SBW25) in relation to other characterised and non-characterised proteins containing the Pfam domain – 03009. A full list of genomes used in the search is provided in Table S5. The evolutionary history was inferred using the Neighbor-Joining method50. The optimal tree with a sum of branch length = 17.19752950 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method and are in the units of the number of amino acid differences per site. The analysis involved 144 amino acid sequences. All ambiguous positions were removed for each sequence pair. There were a total of 483 positions in the final dataset. Evolutionary analyses were conducted in MEGA651. Bootstrap values were omitted for clarity.

Figure 2. Growth of three Pseudomonas strains on differing organic phosphorus (P) compounds as a sole P source (100μM) using succinate (20 mM) as the carbon source. Results shown are the mean of triplicate cultures. The dashed black line indicates approximate OD600 at T = 0. The dashed grey line segregates the positive and negative controls to the organic P treatments. Asterisks denote a significant (T-test, P < 0.05) reduction in growth rate (Fig. S5). Error bars denote standard deviation. Abbreviations: –P, no added orthophosphate; +P 100μM orthophosphate added; Pch, phosphorylcholine; G3P, sn-glycerol-3-phosphate; GPC, glycerolphosphorylcholine; DSM4166, P. stutzeri DSM4166; SBW25, P. fluorescens SBW25; BIRD-1, P. putida BIRD-1; WT, wild type; ΔglpQ, mutant genotype for the secreted glycerolphosphodiesterase; ΔphoBR, mutant genotype for the master regulator of the phosphate starvation response network; ΔphoX, mutant genotype for alkaline phosphatase.
WT strains grew on G3P, the product of characterised GlpQ-mediated GPC catabolism, and phosphorylcholine (Pch) (Table 1).

In addition to glpQI and glpQII, P. stutzeri DSM4166 and P. fluorescens SBW25 also possess plcP, which encodes an intracellular phospholipase C20, 34–36, and another gene encoding the Pfam03009 domain (PSTAA_2726, PFLU1570). Therefore, to further investigate if GlpQI and GlpQII were responsible for growth on exogenous GPC in both P. stutzeri DSM4166 and P. fluorescens SBW25, respectively, a ΔglpQ mutant was constructed for each strain by marker exchange mutagenesis. Both ΔglpQ mutant variants of P. stutzeri DSM4166 and P. fluorescens SBW25 no longer grew on G3P as a sole source of P. However, both mutant strains could still utilise G3P and Pch (Fig. 2). Plasmid-encoded complementation of either mutant with their respective native glpQ restored growth on GPC (Fig. 2). Furthermore, we also separately introduced the two plasmids harbouring either glpQI or glpQII into the wild type strain of P. putida BIRD-1. Complementation with either glpQ gene conferred the ability of this bacterium to utilise GPC as a sole source of P. Together, these data confirm that glpQI and glpQII encode two phosphodiesterases capable of cleaving phosphorylhead groups.

### Pseudomonas strains perform all the catabolic steps of GPC metabolism extracellularly.

No apparent transporter for the predicted metabolite of GPC cleavage (glycerol 3-phosphate, G3P), namely GlpT nor UgpABCE, is present in the genome of P. stutzeri DSM4166. Therefore we hypothesised that G3P degradation occurs extracellularly via one of its ALPs (PhoXI, PhoXII and PhoD) that are also abundantly secreted in P. stutzeri DSM4166. Therefore, to further investigate if GlpQI and GlpQII were responsible for growth on exogenous GPC in both P. stutzeri DSM4166 and P. fluorescens SBW25, respectively, a ΔglpQ mutant was constructed for each strain by marker exchange mutagenesis. Both ΔglpQ mutant variants of P. stutzeri DSM4166 and P. fluorescens SBW25 no longer grew on G3P as a sole source of P. However, both mutant strains could still utilise G3P and Pch (Fig. 2). Plasmid-encoded complementation of either mutant with their respective native glpQ restored growth on GPC (Fig. 2). Furthermore, we also separately introduced the two plasmids harbouring either glpQI or glpQII into the wild type strain of P. putida BIRD-1. Complementation with either glpQ gene conferred the ability of this bacterium to utilise GPC as a sole source of P. Together, these data confirm that glpQI and glpQII encode two phosphodiesterases capable of cleaving phosphorylhead groups.

### GPC-utilising bacteria can liberate P for the bacterial community.

Based on these results, we propose a model for GPC degradation illustrating how non-GPC degrading bacteria may benefit from its extracellular breakdown (Fig. 3). To test this hypothesis a series of co-cultivation experiments were established with different combinations of various Pseudomonas strains. Initially, the P. stutzeri DSM4166 or P. fluorescens SBW25 wild type were co-cultured with their respective ΔglpQ mutant strains with low Pi (100 μM) or GPC (100 μM) as the sole P source. Although neither ΔglpQ mutant could grow on GPC in isolation (Fig. 2), the mutants did grow in the presence of their parental wild type strains (Fig. 4A). P. fluorescens SBW25 does possess a glpT homolog (identity 74.31%, e-value 0.0e + 00) to E. coli glpT), therefore it was interesting that the corresponding ΔglpQ mutant could still utilise the G3P produced by GlpQ. In addition, when P. putida BIRD-1 (non-GPC utilising) was co-cultivated with wild type P. stutzeri DSM4166 or P. fluorescens SBW25 (GPC-utilising) using GPC as the

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Low Pi</th>
<th>High Pi</th>
<th>Pch</th>
<th>G3P</th>
<th>GPC</th>
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<tr>
<td>P. stutzeri</td>
<td>Wild type</td>
<td>+</td>
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<tr>
<td>DSM4166</td>
<td>ΔglpQI − disrupted glpQI</td>
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<td>ΔphoBR − silenced pho regulon, inc. pstSABC</td>
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<td>ΔglpQI + pBR ΔglpQI − complemented with native glpQI</td>
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<tr>
<td>P. fluorescens</td>
<td>Wild type</td>
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<td>SBW25</td>
<td>ΔglpQI − disrupted glpQI</td>
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<td>ΔglpQI + pBR ΔglpQI − complemented with native glpQI</td>
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<td>P. putida</td>
<td>Wild type</td>
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<tr>
<td>BIRD-1</td>
<td>ΔglpQI − disrupted glpQI</td>
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<td>ΔglpQI + pBR ΔglpQI − complemented with native glpQI</td>
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Table 1. Growth characteristics of the various strains and mutants used in this study with respect to P utilisation. Abbreviations: Pi, orthophosphate; Pch, phosphorylcholine; G3P, glycerol 3-phosphate; GPC, sn-glycerol phosphorylcholine; ALP, alkaline phosphatase; pstSABC, genes encoding the high-affinity Pi transporter. Growth occurred, but the rate was slower than that of the wild type.
resulted in a collection of 462 distinct hits (Fig. S6). Therefore, BLASTP searches returned an order of magnitude more 
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To gain a better understanding of the abundance and subsequent expression of GDPDs in agricultural soils, we screened a number of metagenomes (MG) and metatranscriptomes (MT) generated from the Centre INRS-Institut Armand-Frappier, Laval, Canada, deposited in the IMG/IGI database (Project ID – Gp0115425). Functional searches were performed using certain Pfam domains associated with Pi-scavenging enzymes: Pfam03009 (GDPD), Pfam05787 (PhoX), Pfam00245 (PhoA), as well as various Pfam domains (Pfam00154/10415/00549/00140) associated with genes encoding housekeeping proteins (RecA/SucD/RpoD) as the queries. In order to compare Pi-scavenging function with another important soil function, Pfam domains associated with genes encoding the nitrogen fixation enzymes NifH/Q were also added to the analysis. As expected, in both the MGs and MTs a number of reads were assigned to Pfam domains associated with housekeeping functions. Interestingly, we also found a number of reads associated with ALPs (PhoX, PhoA, PhoD) and GDPDs in both the MGs and MTs whilst only a few reads that were assigned to Pfam domains associated with NifH/Q were retrieved (Fig. 5A). The number of reads associated with GDPDs and ALPs (PhoX, PhoA, PhoD) was slightly greater in the MTs compared to the MGs (Fig. 5A). Indeed, the ratio of Pfam domains associated with NifH/Q were retrieved (Fig. 5A). The number of reads associated with GDPDs and 
Pseudomonas strains. Each cleavage step occurs either in the periplasm or extracellularly resulting in the production of free inorganic orthophosphate (Pi). Cells can then take up the Pi through Pi-specific transporters. Abbreviations: ALPs, alkaline phosphatases; PstSABC, high-affinity Pi transporter; other abbreviations are as stated in the previous figure.

Figure 3. A proposed model for the metabolic steps of glycerolphosphorylcholine (GPC) degradation among Pseudomonas strains. Each cleavage step occurs either in the periplasm or extracellularly resulting in the production of free inorganic orthophosphate (Pi). Cells can then take up the Pi through Pi-specific transporters. Abbreviations: ALPs, alkaline phosphatases; PstSABC, high-affinity Pi transporter; other abbreviations are as stated in the previous figure.
The two major GlpQ producers in these soil environments are *Actinobacteria* and *Proteobacteria*, whilst a number of hits were also assigned to *Firmicutes*, *Gemmatimonadetes*, *Cyanobacteria* and *Acidobacteria* (Fig. S7A). In MGs the ratio between reads assigned to *Proteobacteria* and *Actinobacteria* was 2:1. However, in the MTs this became a 1:1 ratio (Fig. S7A), suggesting that both of these phyla play equally important roles in glycerolphosphodiester degradation in the soil. Based on their relative abundance in the MTs, the *Gamma*- and *Betaproteobacteria* appeared to be the two major classes responsible for glycerolphosphodiester degradation in soils.

Finally, in order to better understand the cycling of phospholipid headgroups in the rhizosphere, we compared bulk soil versus rhizosphere MT samples from the Kellogg Biological Station, Michigan, USA, again using the function search option in IMG/JGI. The number of transcripts assigned to Pfam domains associated with housekeeping genes was comparable (unpaired T-Test, *P* > 0.05) between soil and rhizosphere samples (Fig. 6). There were a greater number of transcripts assigned to PhoA, PhoX and PstS in the rhizosphere, although only PhoX was statistically significant (unpaired T-test, *P* < 0.05). However, there was a statistically significant (unpaired T-test, *P* < 0.05) reduction in the number of transcripts assigned to GDPDs in the rhizosphere compared to bulk soil. This result was also confirmed by BLASTP (cut-off - e-20, 30% identity) analysis using GlpQI from *P. stutzeri* DSM4166 as the query, where 20 hits were retrieved from bulk soil and only 4 were retrieved from the rhizosphere samples.

Taken together, these data reveal that genes involved in liberating Pi from glycerolphospholipid headgroups are abundant, derived from a diverse range of bacterial taxa and are expressed in the soil.

**Discussion**

Whilst non-specific ALP and phosphodiesterase activities (and to a lesser extent phophonate and phytate degradation) have been extensively surveyed in various soils, the activity of GDPDs in soils has received little attention. Using a combination of bioinformatic and genetic approaches we identified and characterised two secreted GDPDs in soil-dwelling/plant associated *Pseudomonas* species essential for growth on exogenous GPC, termed GlpQI (from *P. stutzeri* DSM4166) and GlpQII (from *P. fluorescens* SBW25). Whilst the degradation of phospholipids and their corresponding headgroups in soil is poorly documented, limited evidence suggests that their degradation results in an increase in soil Pi. GlpQI and ALPs are among the most abundant exoenzymes detected in the exoprotoemtes of Pi-depleted *Pseudomonas* and *Bacillus* cells, suggesting that these enzymes...
are likely responsible for this release of Pi from phospholipids. Here, as well as providing genetic evidence for
the function of two distinct *Pseudomonas* GlpQs (Fig. 2), we also performed a series of co-culture experiments
to provide ‘proof-of-concept’ that phospholipid headgroups are degraded extracellularly and that non-GPC
utilising bacteria can benefit from the action of secreted GlpQ (Fig. 4). The fact that only the *Pseudomonas* 
\( \Delta \text{phoBR} \) mutants, unable to express the PstSABC transporter\(^\text{20}\), could no longer grow in co-cultivation with a
GPC-degrader strengthens the hypothesis that Pi is released extracellularly (lower sections of Fig. 4A,C ). In
*E. coli* GlpQ is thought to be localised to the periplasm\(^\text{27}\), so whether *Pseudomonas* GlpQ enzymes are primarily located
in the periplasm & leak outside the cell non-specifically as seen for other periplasmic proteins\(^\text{20, 39, 40}\) or are truly
‘secreted’ proteins, remains to be determined. GlpQ was also an abundant protein in the *Bacillus* exoproteome
during Pi depletion suggesting that this enzyme may be secreted\(^\text{21}\).

*P. fluorescens* SBW25 was isolated from the phytosphere of sugarbeet and is an effective coloniser of the pea
rhizosphere\(^\text{41}\), whereas *P. stutzeri* DSM4166 was isolated from the rhizosphere of yellow indiangrass (*Sorghum
nutans*)\(^\text{33}\). Like other GlpQ-harbouring bacteria, both of these *Pseudomonas* strains form close associations with
eukaryotic organisms suggesting that the ability to degrade phospholipid headgroups confers an ecological
advantage in these environments\(^\text{20, 29, 30, 37}\). Interestingly, in *P. stutzeri* DSM4166, growth on GPC as a sole P source
was also dependent on a functional pho regulatory system as the phoBR knockout mutant failed to grow (Fig. 2).
This observation, coupled with its abundant secretion under Pi depletion\(^\text{17}\) suggests that GPC may be utilised pri-
marily as a source of P in the rhizosphere which is often a limiting substrate for bacteria whose primary nutrient
source is carbon-rich plant root exudates, namely amino acids, organic acids and carbohydrates\(^\text{42}\).

Figure 5. The abundance of various genes and their corresponding transcripts encoding Pfam domains
associated with various phosphate (Pi)-scavenging (pink diamonds), nitrogen fixing (green diamonds) and
housekeeping functions (blue diamonds) sampled at the Centre INRS-Institut Armand-Frappier, Laval, Canada
(A). The mean (n = 16) number of reads assigned to each Pfam domain retrieved from either the metagenomes
(MG) or metatranscriptomes (MT). Error bars denote standard deviation. The number of reads assigned to
various Pfam domains associated with Pi-scavenging were plotted against the number of reads assigned to Pfam
domains associated with RpoD (B), SucD (C), RecA (D). Abbreviations: ALPs, alkaline phosphatases; GDPDs,
glycerolphosphodiesterase).
Whilst GlpQI in P. stutzeri DSM4166 was abundantly secreted in response to Pi-depletion, GlpQII in P. fluo-
srescens SBW25 was not among the most abundant exoproteins found in its exoproteome, but was still responsive
to Pi depletion. This observation may be attributed to differences in regulatory capacity in these two strains.
However, both glpQI and glpQII are found in the genome of P. aeruginosa and in this bacterium glpQI showed a
greater up-regulation (25-fold) compared to glpQII (5.8-fold) in response to Pi-depletion. A similar regulatory
pattern was observed in Mycoplasma pneumoniae, with the suggestion that the two GlpQ enzymes may have
differing substrate specificities and physiological roles within the cell. Although not investigated in this study,
expression of GlpQs is induced/repressed by carbon sources and carbon limitation, which may also affect
the levels of Pi released from phospholipids in soils.

Whilst recent studies have addressed the distribution of ALPs in soil, the distribution of GDPDs, including
GlpQ, in this environment is unknown. Although phospholipids are ubiquitous in bulk soil, we hypothesised
that their abundance may be higher in the rhizosphere due to plants releasing mucilage that is partly composed
of phosphatidylcholine. Furthermore, the rhizosphere is often Pi-depleted which may lead to induction of glpQ
akin to the induction of ALP activity. Subsequently, we predicted that an enrichment of the genes encoding
GDPDs, and their corresponding transcripts, may be higher in the rhizosphere compared to bulk soil. However,
whilst we found no evidence that genes encoding GDPDs are differentially transcribed in the rhizosphere
and bulk soil, the number of transcripts assigned to ALPs (PhoX and PhoA) was higher in the rhizosphere.
Indeed, phosphatase activity (at pH 6.5) was higher in P. putida BIRD-1 cells retrieved from the rhizosphere
compared to cells retrieved from the bulk soil indicating an induction of PhoX or similar exoenzymes. Interestingly,
glycerolphosphorylethanolamine, which is generally the major head group of phospholipids found in bacteria,
was also a substrate for E. coli GlpQ. This may help explain the abundance of glpQ in bulk soil samples. Therefore, investigating the substrate specificity of various soil-dwelling GlpQ enzymes warrants future attention.

It appears that multiple phyla are contributors to phospholipid head group degradation. The genomes of
Actinobacteria often contain multiple forms of glpQ whilst Proteobacteria, generally contain only one copy
of glpQ and a putative ugpQ. Betaproteobacteria, which are frequently enriched in the rhizosphere of plants,
also appear to be important recyclers of glycerolphosphodiester, as both MGs and MTs had a number of hits related
to this class (Fig. S7). Inspection of genomes deposited in the National Centre for Biotechnology information

Figure 6. The number of transcripts encoding Pfam domains associated with various phosphate (Pi)-
scavenging and housekeeping functions detected in the metatranscriptomes (MT) sample at the Kellogg
Biological Station, Michigan, USA. Results shown are the mean of triplicate cultures. Error bars denote standard
deviation. Pfam03009, glycerolphosphodiester phosphodiesterase; Pfam05787, alkaline phosphatase–PhoX;
Pfam00245, alkaline phosphatase–PhoA; Pfam16655, alkaline phosphatase–PhoD; Pfam12849, high-affinity
Pi substrate binding protein–PstS; Pfam00549, SucD; Pfam00154, RecA; Pfam00012, DnaK.
In conclusion, the current study provides evidence for a mechanism to explain the observed Pi release from phospholipid degradation in soils. Using *Pseudomonas* as a model, our data demonstrates how the extracellular cleavage of GPC and its breakdown products can cross-feed P into the wider microbial community and likely help plants acquire more Pi. Finally, the capacity for this potential plant-growth promoting effect is not confined to *Pseudomonas*, but is present in various phylogenetically distinct soil bacteria.

**Materials and Methods**

**Bacterial strains and growth conditions.** *Pseudomonas putida* BIRD-1, *P. fluorescens* SBW25, and *P. stutzeri* DSM4166 were maintained on Luria Bertani (LB) agar (1.5% w/v) medium at 30 °C. Their respective mutants were maintained on similar plates containing the appropriate antibiotic. For all growth experiments triplicate cultures were grown in an adapted Minimal A medium comprising: Na-Succinate 5.4 g L\(^{-1}\), NaCl 200 mg L\(^{-1}\), NH\(_4\)Cl 450 mg L\(^{-1}\), CaCl\(_2\) 200 mg L\(^{-1}\), KCl mg L\(^{-1}\), MgCl\(_2\) 450 mg L\(^{-1}\), FeCl\(_3\) 10 mg L\(^{-1}\), MnCl\(_2\) 10 mg L\(^{-1}\), 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.2. KH\(_2\)PO\(_4\), Pch, G3P, or GPC were added to a final concentration of either 100 μM or 200 μM. *Pseudomonas* species were pre-cultured in minimal A medium containing 100–200 μM Pi to ensure cells had adequate Pi for growth whilst minimising the potential for carry over of residual Pi into triplicate experimental cultures.

**Co-cultivation experiments with various *Pseudomonas* strains.** Starter cultures for each species contained minimal A medium supplemented with 100 μM Pi and incubated overnight. Each strain was inoculated (2%) in an approximate 1:1 ratio (n = 3). The number of colony forming units (CFU) were determined by plate counting (n = 3) at T0 and again (T1) after overnight incubation at 30 °C. LB agar was used to determine bacterial counts and additions of gentamicin or chloramphenicol were used as appropriate to select for mutants and or *P. putida* BIRD-1 wild type. Where no antibiotics could be used, colony morphology was used to distinguish organisms (DSM4166: BIRD-1). The number of generations was determined by calculating the change in CFUs between T0 and T1.

**Generation of the various *Pseudomonas* mutants.** To construct the various *Pseudomonas* mutants, we used the methods developed by Lidbury et al.\(^{20}\) that were previously employed to construct the *P. putida* BIRD-1 ΔphoBR mutant. A full list of strains, plasmids and primers used in this study is outlined in Tables S2 and S3. Initially, two regions of genomic DNA, one at the 5’ end and the other at the 3’ end of the given functional gene were PCR amplified, along with the gentamicin resistance cassette from p34S-Gm\(^{9}\). The suicide vector pK18mobSacB\(^{40}\) was linearised using the restriction enzymes BamHI and HindIII and all four fragments of DNA were ligated together using the HiFi DNA Assembly Kit (New England Biolabs, Hitchin, UK) according to the manufacturer’s guidelines. The resulting plasmid was transformed into *Escherichia coli* S17.1 via electroporation and mobilized into the respective *Pseudomonas* strains via conjugation (3 h at 30 °C) on a 0.22 μm pore-size, 47 mm sterile filter (Millipore, UK), using LB as the medium. Transconjugants were selected on LB containing gentamicin (50 μg ml\(^{-1}\)) and using chloramphenicol (10 μg ml\(^{-1}\)) as the counter selection against *E. coli*. A single crossover transconjugant was grown overnight in LB and plated onto LB containing gentamicin and 10% (w/v) sucrose to select for double crossover mutants. Homologous recombination was confirmed by PCR and DNA sequencing.

**Complementation of various *Pseudomonas* mutants.** All the plasmids and primes used for complementation are listed in Tables S2 and S3. Either phoX (BIRD-1), gplQI or gplQII and their 5’ upstream regions (~300 bp) were cloned into the broad host-range plasmid pBBRMC51-km\(^{40}\) using the HiFi DNA Assembly Kit. pBBRIMCS-km was linearised with KpnI and XbaI. Plasmids were transformed into the respective mutants via electroporation (18 Kg cm\(^{-1}\), 200 A resistance, and 25 Ω capacitance). Transconjugants were selected on LB kanamycin (50 μg ml\(^{-1}\)).

**Quantification of alkaline phosphatase activity.** Alkaline phosphatase activity was recorded according to Lidbury et al.\(^{20}\). Briefly, a cell culture (n = 3) was incubated with 20 μL para-nitrophenyl phosphate (pNPP) (final conc. 4 mM) until colour development occurred. The reaction was stopped using 25 μL NaOH (2 mM) and incubated for 10 min. Cell debris and precipitants were removed via centrifugation (2 min, 8,000 × g) prior to spectrophotometry (optical density 405 nm). A standard curve for para-nitrophenol was generated using a range of known concentrations (0, 34.8, 69.5, 139.1, 278.2, 556.4, μg ml\(^{-1}\)).

**Bioinformatic analysis of Pi-liberating genes/proteins.** Pfam domains associated with various proteins of interest were used as the queries for ‘function searches’ against a number of MGs and MTs deposited in the Integrated Microbial Genomes database (IMG/IGI) (https://img.jgi.doe.gov/). A list of MGs and MTs used in this study are outlined in Table S1. The number of hits retrieved for each MG or MT dataset were normalised by adjusting the total number of base pairs to 1 Gbp. BLASTP (cut off values, e-20, min. similarity 40%) was also conducted on each dataset using various characterised GplQ homologs. To assess the diversity of sequences assigned to GplQ, manually curated databases were established by downloading all the protein sequences in the IMG/IGI database annotated as possessing the Pfam03009 domain. A subsequent BLASTP search was performed using a relatively relaxed stringency (e-10). After sequences were assigned to a protein from a specific strain, the higher taxonomic rank of that strain was determined using the NCBI taxonomy browser tool (https://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi).
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Author Contributions
I.L. and A.M. designed and performed the research. IL wrote the manuscript with guidance from D.S. A.M., T.F., G.B., A.J., A.G., M.T., D.S., E.W., and J.H. all reviewed and revised the manuscript.

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